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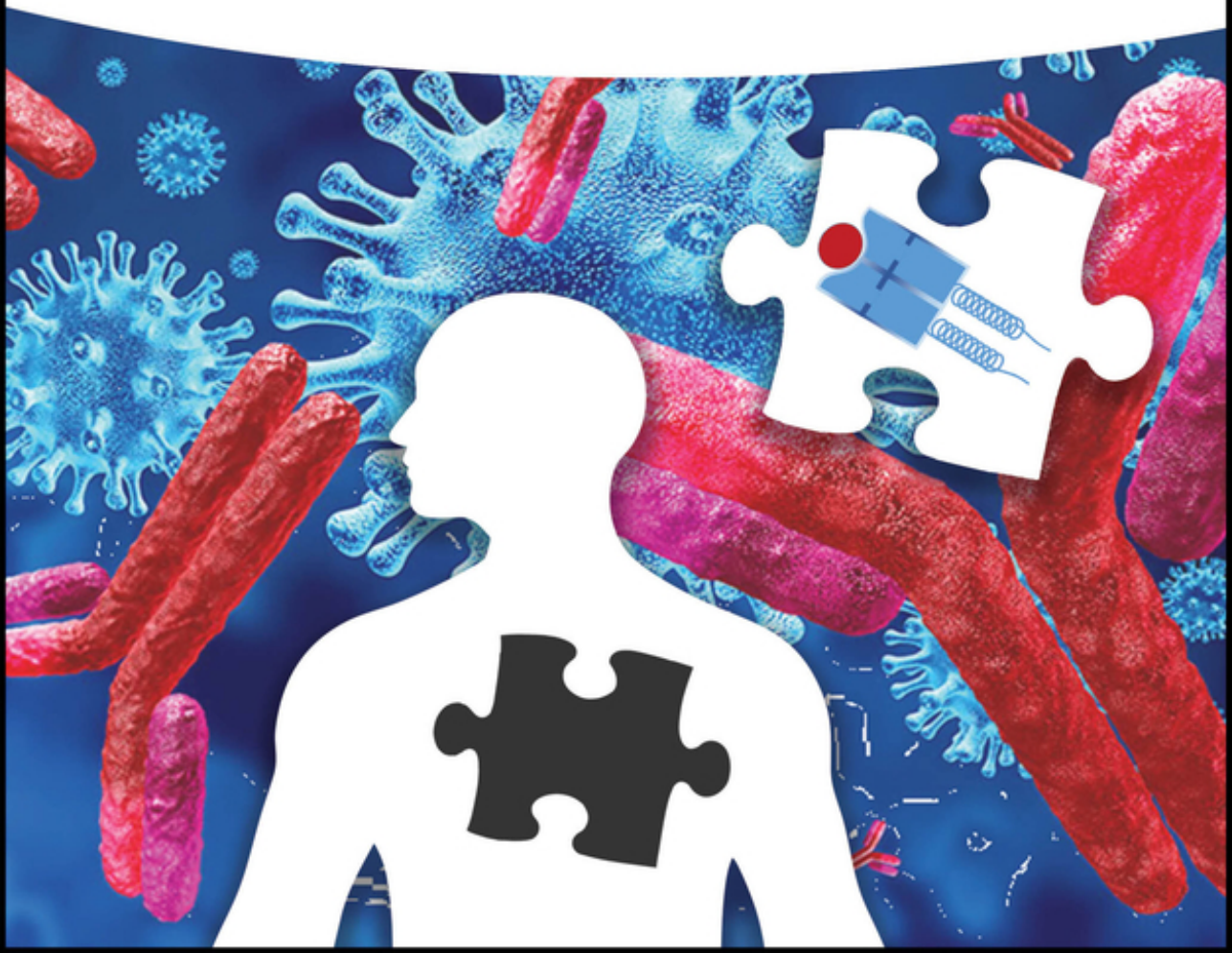
Edited by Volker Oberle

Histocompatibility and Immunogenetics

A Guide for Practitioners



Histocompatibility and Immunogenetics

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Dedicated to my mentors Ulrich Rothe, Dick Hoekstra, Wolfgang Lösche, and Sabine Schröder and to my sons, Oskar, Artur, and Bruno.

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Preface

Dear readers, we are proud to present to you the world's first comprehensive textbook on H&I. This has not been an easy task because there are fields of study that are highly specialized and therefore particularly challenging, as they are at the intersection of much broader disciplines. One such field is H&I, histocompatibility and immunogenetics. Mastering this subject requires the integration of knowledge from immunology, genetics, clinical medicine, and laboratory sciences. Due to the sheer amount of knowledge involved, specific textbooks for these fields are rarely available. This makes it difficult for beginners, clinicians who rely on this knowledge, and interested individuals from other fields to obtain a quick and comprehensive guide to the subject.

During the yearly EFI Congress 2023 in Nantes, where the Education Committee presented a new subject-specific curriculum, the question arose as to what reading materials students should use to learn. Until now, in most cases, teachers have compiled and made available to H&I students only reviews and scientific publications. From a didactic and efficiency point of view, this was certainly not the optimal solution. This made us realize that there was an urgent need for adequate teaching materials. With this in mind, we developed the idea for this innovative textbook.

We are deeply grateful to more than 40 renowned authors from around the world, all experts in their field, who have come together and worked with great enthusiasm and effort to provide a comprehensive overview of H&I. The book covers topics ranging from the basics of polymorphic systems in leukocytes, killer cells, and platelets to their clinical relevance in transplantation and transfusion. It examines the implications for HLA-determined diseases and the laboratory methods needed to study these systems and to diagnose the effects of clinically relevant processes. It integrates these topics in a unique way for the first time.

We are also grateful to Wiley for not only supporting H&I on a scientific level through relevant journals, but also for giving us the opportunity to develop the field didactically with a dedicated textbook.

With this book, we wish readers an educational and engaging experience, whether they are beginning their journey into the field or seeking to broaden their perspective beyond their own area of expertise to gain a deeper understanding of H&I.

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Part I

Biological Basics

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1

Innate Immune Defence

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1.1 Introduction

The innate immune system is the highly evolutionarily conserved first line of defense of the body. It is characterized by its prompt and efficient response involving non-specific mechanisms that may be constitutive, such as physical-chemical barriers and the complement system, or rapidly inducible, such as the inflammatory reaction.

In this chapter, we will begin by describing the anatomical barriers that protect the host against infection and examine the immediate innate defenses provided by various secreted soluble proteins, known as antimicrobial enzymes and peptides. We will then discuss the complement system, which directly kills some microorganisms and interacts with others to promote their removal by phagocytic cells.

Next, we will explore the induced mechanisms of the innate immune response. We will start by detailing the different strategies of danger recognition used by immune cells, with a particular focus on pattern recognition receptors (PRRs), whose discovery has significantly improved our understanding of immune pathophysiology. We will then discuss the inflammatory reaction, outlining the mechanisms involved in the initiation of inflammation and providing an overview of the different phagocytic cells and the steps involved in the process of phagocytosis. Additionally, we will introduce other cells of the innate immune system, known as innate and innate-like lymphoid cells, including natural killer cells (NK cells). These lymphoid cells contribute to innate host defenses against viruses and other intracellular pathogens.

Finally, we will discuss the role of innate immunity in the initiation and polarization of the next phase of the immune response: adaptive immunity.

1.2 Anatomical Barriers and Initial Chemical Defenses

The first phase of host defense, called immediate innate defenses, consists of non-specific mechanisms that are always active and ready to respond to any

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pathogen. Epithelial surfaces act as a physical barrier against microbial colonization and invasion, but also as a chemical barrier, producing a variety of antimicrobial molecules.

1.2.1 Skin and Mucosal Barriers

Anatomical barriers provide the crucial first line of defense by preventing exposure of internal tissues to microorganisms. These barriers include epithelia of the skin on the outer surfaces of the body along with mucosal surfaces of the respiratory, gastrointestinal, and urogenital tracts. Epithelial cells are held together by tight junctions, which effectively form a seal against the external environment [1]. The internal epithelia secrete a viscous fluid called mucus, which contains glycoproteins called mucins, presenting several protective functions. There are two types of mucins, gel-forming and transmembrane. All are characterized by large, highly O-glycosylated mucin domains that are diversely modified by Golgi glycosyltransferases to become extended rodlike structures [2]. The general function of mucus on internal epithelial surfaces is to prevent microorganisms from adhering to the epithelium. In the respiratory tract, microorganisms can be expelled in the outward flow of mucus driven by vigorous mucociliary clearance mechanisms. The latter function is most evident in the large intestine, where the inner mucus layer separates the numerous commensal bacteria from the epithelial cells. Also, in the gut, peristalsis is an important mechanism for keeping both food and infectious agents moving through the body. The extreme susceptibility to infection observed in patients suffering from severe cutaneous burns or primary ciliary dyskinesia demonstrates the great importance of this first barrier and the inability of an intact immune system to compensate for its loss [3].

1.2.2 Antimicrobial Enzymes and Peptides

At anatomic barriers, additional resistance mechanisms participate to further strengthen host defenses. Most epithelia produce a wide variety of chemical substances that are microbicidal or that inhibit microbial growth. For example, the acid pH of the stomach and the digestive enzymes, bile salts, fatty acids, and lysolipids present in the upper gastrointestinal tract create a substantial chemical barrier to infection. One important group of antimicrobial proteins includes lysozyme and secretory phospholipase A2, antibacterial enzymes secreted in tears and saliva that attack the chemical characteristics of bacterial cell walls [3].

The second category of antimicrobial agents secreted by epithelial cells and phagocytes consists of antimicrobial peptides such as defensins, cathelicidins, and histatins. These peptides are secreted by epithelial cells at the mucosal surface and by phagocytes in tissues. Defensins are notably an ancient and evolutionarily conserved class of antimicrobial peptides produced by many eukaryotic organisms, including mammals, insects, and plants. Defensins are short cationic peptides (30–40 amino acids) generated by proteolytic processing from inactive propeptides. They act within minutes to disrupt the cell membranes of bacteria and fungi, as

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well as the membrane envelopes of some viruses. Three subfamilies of defensins (α, β, and θ) are distinguished by their amino acid sequences. Humans only present α- and β-defensins as the human gene homologous to the θ-defensin gene described in rhesus macaques contains a premature stop codon. To date, six human α-defensins have been identified, which are further divided into two major classes according to their expression patterns and gene structures: myeloid defensins or human neutrophil peptides (HNPs) 1–4 and human (enteric) defensins (HDs) 5 and 6. HNPs are stored in the azurophilic granules of human neutrophils, along with several other antimicrobial agents. These peptides are typically directed to fuse with phagolysosomes but can also be released into the extracellular medium through the degranulation of activated neutrophils (see part 1.5). HD5 and HD6 are constitutively expressed and secreted by Paneth cells at the bottom of the small intestinal crypt, contributing to the physiological maintenance of the digestive barrier. Although more than 30 β-defensin genes exist in the human genome, only a few have been extensively characterized at the genomic and functional levels. Their expression is restricted to keratinocytes of the skin and epithelial cells. Human β-defensin 1 (HBD1) is constitutively expressed in various epithelial and mucosal tissues. HBD2 and HBD3 are induced by microbial aggressions and pro-inflammatory cytokines, primarily in epithelia of the respiratory and urogenital tracts, skin, and tongue. β-Defensins produced by keratinocytes in the epidermis and by type II pneumocytes in the lungs are packaged into lamellar bodies, lipid-rich secretory organelles that release their contents into the extracellular space to form a watertight lipid sheet in the epidermis and the pulmonary surfactant layer in the lung [4].

Another type of bactericidal protein synthesized by epithelia is carbohydrate-binding proteins, or lectins. C-type lectins require calcium for the binding activity of their carbohydrate-recognition domain, which provides a variable interface for binding carbohydrate structures. C-type lectins of the RegIII family include several bactericidal proteins expressed by intestinal epithelium in humans and mice. Human RegIIIα (also called HIP/PAP for hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein) preferentially kills Gram-positive bacteria directly by forming a hexameric pore in the bacterial membrane [5].

Finally, peptides S100A8 and S100A9, which heterodimerize to form calprotectin, are produced in high amounts by neutrophils, T cells, and intestinal epithelia. Calprotectin acts to sequester magnesium and iron required by microorganisms and exerts a local antimicrobial effect [5].

Of note, most healthy epithelial surfaces are also associated with a large population of nonpathogenic bacteria, known as commensal bacteria or microbiota. These bacteria help to reinforce the barrier functions of epithelia and can themselves produce antimicrobial substances such as lactic acid by vaginal lactobacilli or bacteriocins by other bacterial strains. Commensal microorganisms also exert an indirect influence by stimulating the epithelial cells to produce antimicrobial peptides. This illustrates how the elimination of commensal microorganisms by antibiotic treatment can pave the way for pathogens to proliferate and cause other diseases [3].

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1.3 The Complement System

The complement system is a highly conserved protein interaction platform of the innate immune system, working in coordination with other effectors of immunity to protect the body against infection and other insults.

The complement system is an enzymatic cascade made up of numerous soluble and membrane proteins involved in immune surveillance both in physiological and pathological conditions. These proteins interact with non-immune cells (epithelial cells, osteoclasts, etc.), innate immune cells (macrophages, dendritic cells, neutrophils, mast cells, eosinophils, basophils), and adaptive immune cells (B and T cells). The complement system has multiple functions. It triggers an immune response to foreign pathogens, has pro-inflammatory activity, regulates cytokines production, and helps to remove immune complexes and dead or modified cells (following injury, hypoxia, after viral infection, or tumor-caused modification) [6].

Complement activation is mediated by finely regulated cascades of proteolytic reactions through three main pathways, each initiated by different stimuli: the classical pathway, the lectin pathway, and the alternative pathway (Figure 1.1).

- The classical pathway (CP) is activated by antibodies once those bind to antigens. When this complex is formed, the fraction fields (Fc) of IgM, IgG3, and IgG1 interact with the collagen-like tail of C1q. C1q is part of the C1 complex, which comprises a single C1q molecule bound to two molecules of each C1r and C1s. Once activated, the C1s enzyme cleaves C4 and then C2.
- The lectin pathway (LP) is activated by the binding of C-type lectin and mannose-binding lectin (MBL), or proteins termed ficolins (L-ficolin, H-ficolin, and M-ficolin), to some carbohydrates on the pathogen surface that are rich in mannose and beta glycans, respectively. MBL and ficolins may form a complex with MBL-associated serine proteases (MASPs) and with a smaller molecule called MAp19. When the MBL complex binds to a pathogen surface, MASP-1 and MASP-2 are activated to cleave C4 and C2.
- The alternative pathway can be initiated by many microbial surfaces. While the classical and lectin pathways are activated exclusively after danger recognition through binding antibodies or proteins, the alternative pathway is also constantly active at a low level at the steady state. It can be activated by spontaneous hydrolysis of C3 to give C3 (H_2O), which binds factor B and enables the bound factor B to be cleaved by factor D.

All pathways converge to the assembly of multi-protein enzymatic complexes termed C3 and C5 convertases. C3 and C5 convertases cleave C3 and C5, respectively, into their bioactive fragments that mediate a wide array of functions on complement receptor-expressing cells [2, 7].

There are various types of C3 convertase, depending on the complement pathway activated. In the classical and lectin pathways, the C3 convertase is formed from membrane-bound C4b complexed with C2b (C4b2b). In the alternative pathway, a homologous C3 convertase is formed from membrane-bound C3b complexed with Bb (C3bBb). The alternative pathway can act as an amplification loop for all

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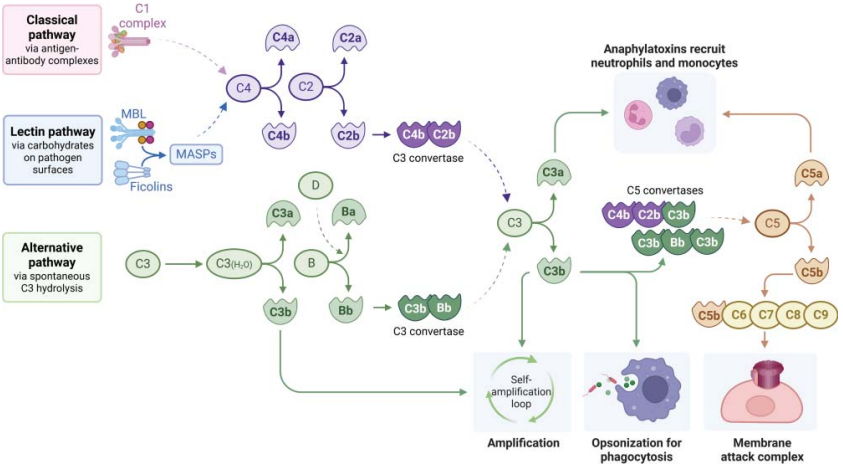

Figure 1.1 The complement system. The complement cascade is schematized here, with a highlight on the three activation pathways. All of them lead to C3 molecule cleavage, which is a key factor in the complement action. During the complement cascade, small peptides named anaphylatoxins are secreted, which contribute to the recruitment of immune cells. C3b coats the microbial or apoptotic cell surface, and the cascade can progress, while C5b starts the terminal pathway. Once C5b is released, it binds to C6 on the target cell surface, where other components of the MAC will join them. Source: "Created with BioRender.com".

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three pathways, as it is initiated by the binding of C3b. C3 is a key mediator in the activation of all complement activation pathways. Once C3 is cleaved by a C3 convertase, it is divided into C3a and C3b. C3a is an anaphylatoxin. It plays a role in innate immune cell recruitment and has antifungal and antimicrobial activity [8]. C3b and some other complement cleavage products belong to the family of opsonins, which can bind to the surface of pathogens and mark them for phagocytosis by immune cells. This opsonization improves the efficiency of pathogens' elimination by phagocytic cells.

C3b also binds the C3 convertase to form a C5 convertase (C4b2b3b or C3bBb3b) that cleaves C5 into C5a and C5b. C5a, just like C3a, is an anaphylactic peptide that attracts and activates neighboring cells to release inflammatory mediators. On the other hand, C5b initiates the "late" events of complement activation called the terminal pathway. It leads to the formation of the membrane attack complex (MAC), which is a cell membrane-perforating complex derived from the assembly of C5b to C9 components (C5b6789). It induces osmotic changes in the target cell through the entry of water, ions, and other molecules. This will lead to cell lysis, and the production of cytokines, prostaglandins, and other pro-inflammatory molecules [2, 7].

The complement system is strictly regulated to prevent inappropriate self-directed activation and tissue damage. Most of these regulatory proteins belong to the regulators of complement activation (RCA) gene family. This family includes soluble regulators such as factor H and C4b-binding protein (C4BP) as well as membrane-bound regulators, such as membrane cofactor protein (MCP or CD46), complement receptor type 1 (CR1), and the glycosylphosphatidylinositol (GPI)-anchored protein decay-accelerating factor (DAF or CD55) [9].

Of note, the complement system can also regulate tolerance to self-antigens and influence adaptive immunity by promoting antigen presentation, activating T and B cells, and regulating antibody production [10]. Other physiological functions attributed to complement are angiogenesis, mobilization of hematopoietic stem/progenitor cells, lipid metabolism, coagulation pathway, calcium metabolism, organ regeneration, and neuroprotection (including migration of neurons and synapse elimination).

1.4 Danger Recognition

Beyond immediate immune defenses, the next phase of the innate immune response is to induce an inflammatory response to recruit new phagocytic cells and circulating effector molecules to the site of infection. This process involves the rapid detection of "immunological danger" through different mechanisms.

1.4.1 Pattern Recognition Receptors (PRRs)

Innate immunity relies upon a limited repertoire of germline-encoded invariant receptors called PRRs. First, PRRs recognize "microbial non-self" represented by conserved molecular structures expressed by a large variety of microbes and

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grouped together under the term pathogen-associated molecular patterns (PAMPs). These structures include mannose-rich oligosaccharides, peptidoglycans, and lipopolysaccharides of the bacterial cell wall, as well as unmethylated CpG DNA common to many pathogens. Secondly, "damaged self"-derived host molecules that indicate cellular infection, stress, or transformation can also be recognized by some innate receptors. Such indicator molecules have been termed damage-associated molecular patterns (DAMPs), and some of them can be recognized by PRRs. DAMPs represent common metabolites and mediator molecules that are upregulated and released during the cell lysis and tissue damage that occurs in the context of both infectious and sterile inflammation [11]. As an example, in the context of organ transplantation, the release of DAMPs by graft cells under conditions of hypoxia and redox stress triggers the innate immune response, contributing to the pathogenesis of early peri-transplant ischemia-reperfusion injury [12].

Currently, four different classes of PRR families have been identified. These families include transmembrane proteins such as the Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), as well as cytoplasmic proteins such as the retinoic acid-inducible gene (RIG-I)-like receptors (RLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs). These PRRs are expressed in macrophages and dendritic cells, but also in various other immune and non-immune cells. Except for some NLRs, the sensing of PAMPs or DAMPs by PRRs upregulates the transcription of genes involved in inflammatory responses. These genes encode proinflammatory cytokines, type I interferons (IFNs), chemokines, and antimicrobial proteins; proteins involved in the modulation of PRR signaling; and many uncharacterized proteins. The expression patterns of inducible genes differ according to activated PRRs [11].

1.4.1.1 Toll-like Receptors (TLRs)

The TLR families are transmembrane glycoproteins responsible for sensing invading pathogens outside of the cell and in intracellular endosomes and lysosomes. TLRs are characterized by an extracellular region containing N-terminal leucine-rich repeats (LRRs), which are responsible for the recognition of specific ligands and perform extracellular pattern recognition. The cytoplasmic domain contains the same Toll/IL-1R (TIR) domain as IL-1R, which plays a role in signal transduction. To date, 10 functional TLRs (TLR1–10) have been found in humans. Different TLRs recognize the different molecular patterns of microorganisms and self-components (Table 1.1). The cellular localization of TLRs determines the types of ligands recognized and the recognition mechanism [2, 13].

Some TLRs (TLR1, 2, 4, 5, 6, 10) are expressed on the cell surface as heterodimers or homodimers and mainly recognize the membrane components of pathogenic microorganisms, such as lipids, lipoproteins, and proteins. Mammalian TLR1, TLR2, and TLR6 are, for example, cell-surface receptors that are activated by various ligands, including lipoteichoic acid and the diacyl and triacyl lipoproteins of Gram-negative bacteria. They are found in macrophages, dendritic cells, eosinophils, basophils, and mast cells. Ligand binding induces heterodimerization of TLR2 and TLR1 or TLR2 and TLR6. TLR4 recognizes lipopolysaccharide (LPS)

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Table 1.1 PRRs and their main ligands.

Receptor	Localization	Ligand	Source of the ligand
		Toll-like receptors	
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria, fungi, mycobacterium
TLR3	Endosome	dsRNA	Viruses
TLR4	Plasma membrane	LPS	Bacteria
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR7	Endosome	ssRNA	Viruses
TLR8	Endosome	ssRNA	Viruses
TLR9	Endosome	CpG-DNA	Viruses, bacteria, parasites, self
TLR10	Plasma membrane	Unknown	Unknown
		C-type lectin receptors	
Dectin-1/2	Plasma membrane	β-Glucan, α-mannan	Fungi
		RIG-I-like receptors	
RIG-I	Cytosol	Short dsRNA, 5′-triphosphate RNA	Viruses
MDA5	Cytosol	Long dsRNA	Viruses
		NOD-like receptors and inflammasomes	
NOD1	Cytosol	iE-DAP	Bacteria
NOD2	Cytosol	MDP	Bacteria
NLRP3	Cytosol	Ion flux, organelle dysfunction, nucleic acids	Bacteria, viruses, self
		cGAS-STING pathway	
cGAS	Cytosol	dsDNA	Viruses, bacteria, parasites, self

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together with myeloid differentiation factor 2 (MD2) on the cell surface. LPS is a component derived from the outer membrane of Gram-negative bacteria and is known to cause septic shock in the context of uncontrolled bacterial infection. In this case, LPS triggers an excessive secretion of cytokines, particularly tumor necrosis factor α (TNFα), leading to systemic vascular permeability – a side effect of its usual role in containing local infections. Structurally, two complexes of TLR4-MD2-LPS interact symmetrically to form a TLR4 homodimer. TLR4 is also involved in the recognition of viruses by binding to viral envelope proteins and DAMPs (endogenous oxidized phospholipids). TLR5 is expressed on the cell surface of macrophages, dendritic cells, and intestinal epithelial cells. It recognizes flagellin, a protein subunit of bacterial flagella. It is activated only by monomeric flagellin, which is produced by the breakdown of flagellated bacteria in the extracellular space [2, 13, 14].

Other TLRs (TLR3, 7, 8, 9) are located intracellularly in the membrane of endosomes and expressed as homodimers, which recognize nucleic acids derived from viruses and bacteria as well as endogenous nucleic acids in pathological contexts. TLR3, for example, is expressed by macrophages, conventional dendritic cells, and intestinal epithelial cells. It recognizes double-stranded RNA (dsRNA), which is a replicative intermediate of many types of viruses. TLR7, TLR8, and TLR9 are also endosomal nucleotide sensors involved in the recognition of viruses. TLR7 and TLR9 are expressed by plasmacytoid dendritic cells, B cells, and eosinophils. TLR8 is expressed primarily by monocytes and macrophages. TLR7 and TLR8 are activated by single-stranded RNA (ssRNA), which is a component of healthy mammalian cells (but normally confined to the nucleus and cytoplasm and not present in endosomes) and of many virus genomes. In abnormal settings, TLR7 can be activated by self-derived ssRNA from apoptotic cells, and polymorphisms in the human TLR7 gene have been associated with an increased risk of the autoimmune disease systemic lupus erythematosus. TLR9 recognizes unmethylated CpG dinucleotides. In mammalian genomes, CpG dinucleotides in genomic DNA are heavily methylated on the cytosine by DNA methyltransferases. However, in the genomes of bacteria and many viruses, CpG dinucleotides remain unmethylated and represent another PAMP [2, 13–15].

Signaling by mammalian TLRs in various cell types induces a diverse range of intracellular responses that together result in the production of inflammatory cytokines, chemotactic factors, antimicrobial peptides, and antiviral cytokines IFNα and IFNβ from the type I IFNs family. The signaling pathways of TLRs depend on the nature of the stimulus, the specific TLR activated, and the downstream adaptor molecule involved. The two main signaling adaptor molecules associated with mammalian TLRs are MyD88 (myeloid differentiation factor 88) and TRIF (TIR domain-containing adaptor-inducing IFNβ). The MyD88-dependent pathway is utilized by all TLRs except TLR3 and leads to the production of inflammatory cytokines such as pro-interleukin-1β (pro-IL-1β), interleukin-6 (IL-6), and TNFα through activation of the transcription factor nuclear factor-κB (NFκB) or through the mitogen-activated protein (MAP) kinase pathway. TLR7 and TLR9 signaling also triggers the production of type I IFNs in a MyD88-dependent manner, along

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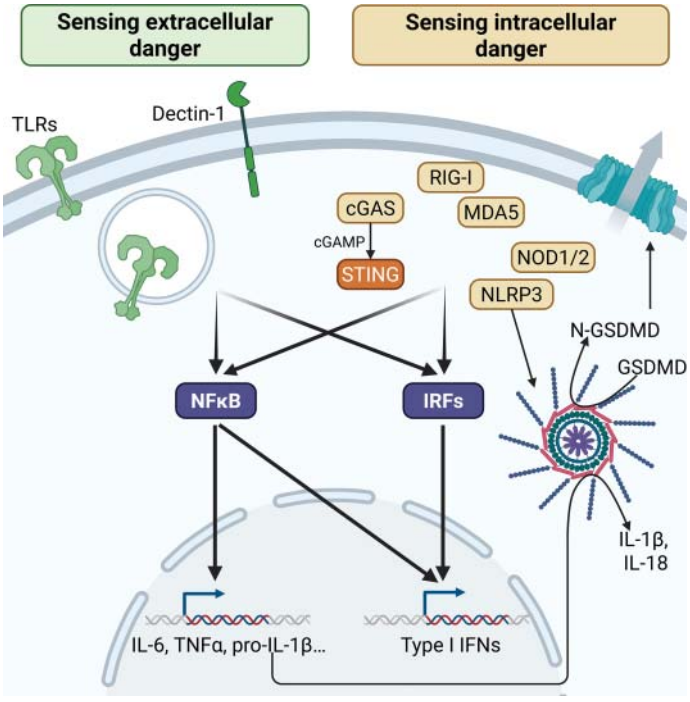

Figure 1.2 PRR signaling. TLRs and CLRs expressed on the plasma membrane (or in endosomes) detect extracellular danger, while cytosolic receptors such as RLRs, NLRs, and cGAS detect intracellular danger. Upon activation, PRRs stimulate transcription factors, including NFκB and IRFs. These transcription factors translocate to the nucleus to activate the expression of genes encoding pro-inflammatory cytokines (such as IL-6, TNFα, pro-IL-1β, and others) or type I IFNs. Some cytokines are then matured through the inflammasome, which itself results from the activation of a specific subtype of NLRs. The inflammasome is also responsible for the cleavage of gasdermin D (GSDMD), which promotes pore formation in the cell plasma membrane and pyroptosis. The cytokines, IFNs, and DAMPs produced will interact in an autocrine and paracrine manner to recruit and activate the effector cells of the innate immune response. Source: "Created with BioRender.com".

with other NFκB-dependent cytokines. Conversely, the TRIF-dependent pathway is used by TLR3 and TLR4 and is associated with the production of inflammatory cytokines through NFκB signaling as well as the production of type-I IFNs through interferon regulatory factors (IRFs) signaling (Figure 1.2). The collective ability of TLRs to activate both NFκB and IRFs means that they can stimulate either antibacterial or antiviral responses as needed [11].

1.4.1.2 C-type Lectin Receptors (CLRs)

CLRs are a family of phagocytic PRRs expressed on macrophages, dendritic cells, and certain tissue cells. They recognize carbohydrates on the surface of self and non-self structures through their carbohydrate recognition domain (CRD) in a calcium-dependent manner (C-type). Depending on the location of the protein on the cell membrane, CLRs are divided into secretory and transmembrane receptors.

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The main representative of secretory receptors is the collagen lectin family, including MBL, that circulates in blood and extracellular fluids and can activate complement (see parts 1.2 and 1.3).

Transmembrane receptors can be divided into type I and type II according to their structure: the N-terminal of type I receptors points to extracellular and contains multiple CRDs, while the N-terminal of type II receptors points to intracellular and contains only one CRD. The dendritic cell-associated C-type lectins (Dectin-1 and -2) are typical representatives of membrane CLRs and recognize polysaccharide structures (glucans, mannans) present in the cell walls of yeast and fungi (Table 1.1). When binding to their ligand, membrane CLRs generate an intracellular activation signal and endocytosis into a cytoplasmic vesicle for direct digestion and elimination.

The CLR family is highly diverse, and the ligands of many of them are not well characterized. Of note, it has been shown that the vast majority of CLRs are involved in the presentation of antigens as active membrane-associated receptors [2, 11, 13].

1.4.1.3 RIG-I-like Receptors (RLRs)

RLRs represent a family of cytosolic PRRs, primarily composed of the retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) receptors. Expressed directly in the cytoplasm, they sense viral RNAs produced within a cell (in contrast to TLR3, 7, 8, 9, which detect extracellular viral RNAs and DNAs that enter the cell from the endocytic pathway).

RIG-I recognizes relatively short dsRNA (up to 1 kb), while MDA5 detects long dsRNA (more than 2 kb). In addition, RIG-I detects the unmodified 5′-triphosphate RNA end of viral ssRNA, which differs from the capped 5′-triphosphate RNA end of self ssRNA also present in the cytosol (Table 1.1). RIG-I and MDA5 bind to viral RNAs using an RNA helicase-like domain in their C-terminal part. They also contain two N-terminal caspase recruitment domains (CARDs) that, when activated, interact with adaptor proteins called mitochondrial antiviral signaling proteins (MAVS) attached to the outer mitochondrial membrane. The signal causes activation of transcription factors IRF3 and IRF7 to produce anti-viral type-I IFNs as well as NFκB to produce pro-inflammatory cytokines (Figure 1.2). In some cell types, RIG-I, but not MDA5, interacts with apoptosis-associated speck-like protein containing a CARD (ASC), thereby inducing the inflammasome, which contributes to inflammatory responses [16].

1.4.1.4 NOD-like Receptors (NLRs) and the Inflammasome

NLRs are another family of cytosolic PRRs. NLRs are composed of three domains: the central nucleotide-binding domain (NBD) for nucleic acid binding and oligomerization of NLRs, LRRs at the C-terminus for ligand recognition, and the N-terminal effector domain, which is the protein interaction domain. According to the different N-terminal effector domains, the NLR family can be divided into five subfamilies:

- NLRC subfamily, which contains CARDs;
- NLRP subfamily, which contains pyrin domains (PYDs);

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- NLRB subfamily, which contains baculovirus inhibitor of apoptosis protein repeats;
- NLRA subfamily, which contains acidic activation domains;
- NLRX subfamily containing other NLR effector domains.

Some NLRs activate NFκB to initiate the same inflammatory responses as the TLRs. It is the case of the most extensively studied NLRs, which are nucleotide-binding oligomerization domain-containing proteins 1 and 2 (NOD1 and NOD2), from the NLRC subfamily (Figure 1.2). NOD proteins recognize fragments of bacterial cell-wall peptidoglycans: γ-glutamyl diaminopimelic acid (iE-DAP) for NOD1 and muramyl dipeptide (MDP) for NOD2 (Table 1.1). When NOD1 or NOD2 recognizes its ligand, it recruits a CARD-containing kinase, which results in activation of NFκB signaling, inducing the expression of genes for inflammatory cytokines and for enzymes involved in the production of nitric oxide (NO) (Figure 1.2). NOD1 is an important activator of responses against bacterial infections and may also function as a systemic activator of innate immunity. NOD2 seems to have a more specialized role, being strongly expressed in the Paneth cells of the gut, where it regulates the expression of potent antimicrobial peptides such as the α- and β-defensins [17].

Other NLRs, as the NLRP subfamily, trigger a distinct pathway that forms multimeric protein complexes called inflammasomes, leading to cell death and the production of pro-inflammatory cytokines (Figure 1.2). The most well-characterized inflammasome is induced through NLRP3. The NLRP3 inflammasome can be activated through three pathways: canonical, non-canonical, and alternative pathways. The canonical NLRP3 inflammasome activation is a two-step process with a priming step relying typically on TLR stimulation and an activation step mediated by an array of stimuli, including ATP, nigericin, gramicidin, bacterial toxins, or particulate matter. So the NLRP3 inflammasome can be triggered either by pathogens or cell damage. NLRP3 activation leads to the recruitment and oligomerization of an adaptor ASC protein, which interacts with the CARD of pro-caspase 1. After assembly, the NLRP3 inflammasome enables the proteolytic activation of caspase 1. This protease promotes the processing and release of mature IL-1β and IL-18, as well as the cleavage of gasdermin D (GSDMD), which promotes pore formation in the cell plasma membrane, leading to a lytic form of cell death called pyroptosis. This process releases various intracellular molecules (ATP, small and large DAMPs such as HMGB1, etc.) that also contribute to inflammation [18].

1.4.2 cGAS-STING Pathway

In addition to TLR9 for extracellular CpG DNA detection, the major intracellular sensor of DNA that triggers the innate immune response is cyclic guanosine monophosphate (GMP)-adenosine monophosphate (AMP) (cGAMP) synthase (cGAS). It senses cytosolic DNA from diverse classes of pathogens that contain DNA or generate DNA in their life cycles (DNA viruses, retroviruses, bacteria, and parasites) as well as self-DNA (genomic and mitochondrial DNA) that can

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enter the cytosol in response to certain cellular stress or environmental insults (Table 1.1). Thus, the cGAS pathway is also involved in "microbial non-self" and "damaged self" recognition. Beyond host defense, this pathway has been shown to play an important role in inflammatory diseases, antitumor immunity, and cellular senescence [19].

The binding of DNA to cGAS induces a conformational change and the generation of the second messenger cGAMP from adenosine and guanosine triphosphate (ATP and GTP) substrates. Then, cGAMP activates the stimulator of interferon genes (STING) at the membrane of the endoplasmic reticulum, which activates a signaling cascade leading to the activation of IRF3 and NFκB and the expression of type-I IFNs and inflammatory cytokines [20] (Figure 1.2).

1.4.3 "Missing Self" and Allorecognition Receptors

Another innate immune recognition strategy involves innate immune receptors that can detect "missing self," i.e. molecules constitutively expressed by normal healthy cells but not expressed by infected, damaged, or transformed cells (or expressed as a variant form in allogenic cells). Recognition of these signals usually delivers an inhibitory signal to prevent activation of the immune response against host tissues, and their absence can thus trigger innate immunity (Figure 1.3).

The inhibitory system is particularly well documented concerning natural killer (NK) cells: inhibitory killer cell immunoglobulin-like receptors (KIRs) that recognize self-major histocompatibility complex (MHC) class I molecules notably play a central role in missing-self recognition by NK cells. This mechanism prevents NK

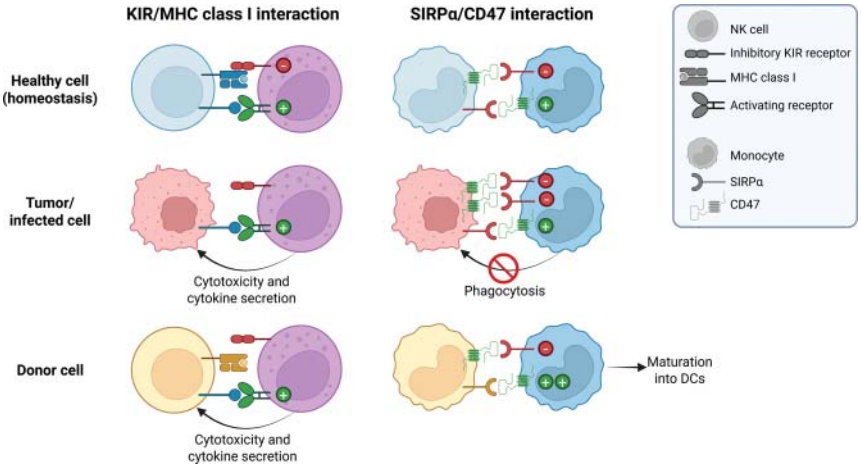

Figure 1.3 Missing self and allorecognition models. Under steady-state conditions, NK cells (KIR/MHC class I interaction model, left panel) or monocytes (SIRPα/CD47 interaction model, right panel) receive equal stimulatory and inhibitory signals from their receptors. Under pathological conditions or in the context of allogenic transplantation, the imbalance between stimulatory and inhibitory signals leads either to cell inhibition or activation (with different effector mechanisms). Source: "Created with BioRender.com".

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cells from killing normal host cells and drives them to target infected/tumor cells that downregulate their MHC class I proteins [21]. More precisely, it is the balance between activating and inhibitory signals that together control NK cells' cytotoxic activity and cytokines production and determine whether an individual NK cell will be triggered to kill a particular target cell (see part 1.5 and Chapter 5).

Another inhibitory receptor of growing interest is the signal regulatory protein α (SIRPα, also known as CD172a). SIRPα is a polymorphic immunoglobulin superfamily receptor expressed mainly on myeloid cells, including macrophages, dendritic cells, and neutrophils. It binds to the ubiquitously expressed CD47 protein. CD47 binding to SIRPα delivers an inhibitory signal that represses phagocytosis by macrophages and inhibits multiple aspects of dendritic cell activation. This mechanism prevents macrophages from engulfing normal host cells, and it is often mentioned as a "do-not-eat-me" signal. In some contexts, this interaction is bidirectional with SIRPα and CD47 engaging each other in opposite directions to deliver stimulatory and inhibitory signals. This is the case in immune cells where SIRPα binding to CD47 has stimulatory functions. It provides costimulatory signals to T cells and enhances dendritic cell homeostasis and migration. Signaling through the CD47-SIRPα axis plays a role in various homeostatic processes, such as the maintenance of erythrocytes, innate immune cells, and T cells. CD47 expression varies according to immune status or pathological condition. Notably, tumor cells and virus- or bacteria-infected cells can upregulate CD47 protein expression to prevent their clearance by macrophages [22, 23].

Of note, in the context of transplantation, these receptors are considered crucial for the mechanisms of innate allorecognition [24–26]. NK cells sense allogenic non-self through the complete absence of KIRs interaction with mismatched non-self MHC class I molecules, whereas monocytes sense subtle variations in the binding affinity of mismatched non-self SIRPα to CD47 (Figure 1.3).

1.5 Inflammatory Reaction and Effector Cells of the Innate Immune Response

Processes activated upon PRR engagement are rapid, considered to be non-specific, and include mechanisms such as phagocytosis, cell locomotion, killing of pathogens or cells, and cytokines production. These innate immune mechanisms are usually very effective in eliminating invading pathogens.

The inflammatory reaction is a complex immune system response to various stimuli, such as infection, injury, or irritants. The effector mechanisms of this reaction can be divided into several key stages (Figure 1.4). Cytokines and chemokines are secreted by inflammatory and damaged cells, attracting leukocytes to the affected area. Inflammation usually involves vasodilation of blood vessels in the affected area. This allows an influx of blood, resulting in local redness and heat, followed by an increase in vascular permeability, which allows plasma proteins, such as antibodies and clotting factors, as well as immune cells, to leave the vessels and reach the site of inflammation. Neutrophils are often the first

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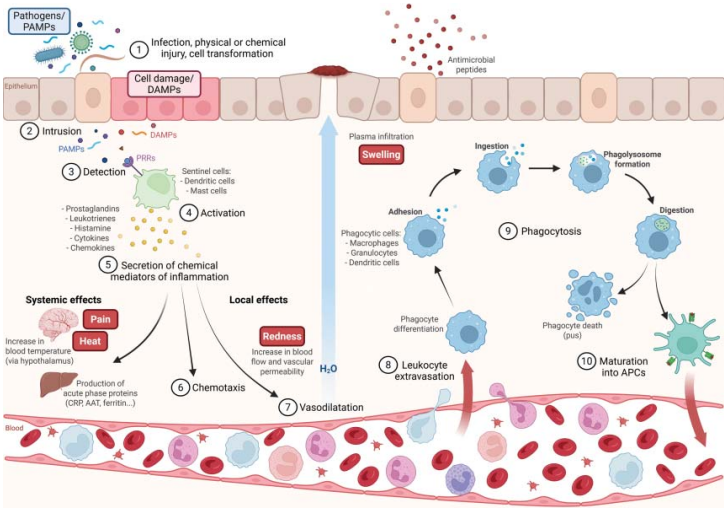

Figure 1.4 The inflammatory reaction. (1–3) Inflammation is induced when host myeloid cells detect PAMPs or DAMPS through germline-encoded PRRs. (4, 5) Cellular stimulation triggers inflammatory processes through the release of various proinflammatory mediators, including prostaglandins, leukotrienes, histamine, cytokines, and chemokines. When released in large amounts, they can exert systemic effects, such as induction of acute-phase proteins in the liver, coagulation activation, or fever. (6–8) Locally, they recruit additional immune cells and activate endothelial cells, thus increasing vascular permeability and facilitating the entrance of attracted immune cells into tissues, but they can also lead to capillary leakage, vasodilation, and hypotension. (9, 10) Attracted cells include activated granulocytes, which exert a crucial role in phagocytosis and pathogen killing, as well as DCs and macrophages, which can mature into APCs. Source: "Created with BioRender.com".

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to arrive, followed by monocytes, which differentiate into macrophages in the tissue. Neutrophils and macrophages are phagocytic cells that engulf and digest pathogens, dead cells, and cellular debris present in the inflammatory zone. At the inflammatory site, immune cells also release various chemical mediators, such as prostaglandins, leukotrienes, cytokines, and growth factors. These mediators contribute to the amplification of the inflammatory response, the activation of immune cells, and the modulation of the immune response. Once the pathogen is eliminated and healing begins, the inflammatory response must be regulated to avoid excessive tissue damage. Anti-inflammatory mediators, such as lipoxins and anti-inflammatory cytokines, are produced to reduce inflammation and promote resolution of the inflammatory response. All these effector mechanisms of the inflammatory response work together to eliminate pathogens, repair damaged tissue, and restore normal function to affected tissue.

1.5.1 Cytokines, Chemokines, Leukotrienes, and Prostaglandins

Chemokines are a type of cytokine involved in cell signaling. They play an important role in immune responses by attracting immune cells to the site of infection or injury. Certain cytokines, known as pro-inflammatory cytokines, promote inflammation and strengthen the immune response to fight infection or tissue damage [27].

Pro-inflammatory cytokines are signaling molecules produced by various types of immune cells and other cells in response to inflammatory stimuli. They act synergistically to amplify and regulate the inflammatory response, contributing to the elimination of pathogens, tissue repair, and the restoration of homeostasis. Here are some of the most important pro-inflammatory cytokines:

- Interleukin-1 (IL-1) is produced by a variety of cells, including macrophages and endothelial cells. It stimulates the production of other pro-inflammatory cytokines and prostaglandins and promotes fever.
- Tumor necrosis factor α (TNFα) is produced mainly by activated macrophages and T cells. It plays a central role in inflammation by inducing the production of other pro-inflammatory cytokines, activating endothelial cells, and promoting the migration of leukocytes to sites of inflammation.
- Interleukin-6 (IL-6) is produced by various cell types, including macrophages, endothelial cells, and fibroblasts, in response to inflammation. It stimulates antibody production, promotes T and B cell differentiation, and contributes to the systemic inflammatory response.
- Interleukin-8 (IL-8) is produced by endothelial cells and various inflammatory cells in response to inflammation. It plays a crucial role in recruiting neutrophils to sites of inflammation by stimulating their migration.
- Tumor necrosis factor β (TNFβ) is produced by several cell types, including T lymphocytes. It is involved in regulating the immune response, and exerts pro-inflammatory effects, notably by activating endothelial cells and stimulating the production of other cytokines.
- Interferon γ (IFNγ) is produced predominantly by NK and T cells, and to a lesser extent by other immune cells including DCs and macrophages, in response to

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infections or other stimuli. It plays various roles in modulating the immune response including macrophage and DC activation, upregulation of MHC expression, antigen processing and presentation, antiviral state, immunomodulation, and leukocyte trafficking.

Anti-inflammatory cytokines are important molecules in regulating the immune response and reducing inflammation. They act by modulating the activity of immune cells and inhibiting the production of pro-inflammatory cytokines. Here are some key examples of anti-inflammatory cytokines and their roles:

- Interleukin-10 (IL-10) inhibits the production of pro-inflammatory cytokines such as IL-1, IL-6, and TNFα. IL-10 promotes the differentiation of regulatory T cells and reduces the expression of co-stimulatory molecules on macrophages and dendritic cells. It binds to IL-10R1 and IL-10R2 receptors, activating the JAK1 and STAT3 signaling pathways, leading to anti-inflammatory effects.
- Transforming growth factor-β (TGFβ) plays a crucial role in regulating inflammation and tissue repair, inhibits the proliferation and activation of T and B lymphocytes, and promotes the formation of regulatory T cells. It binds to TGF-βR1 and TGF-βR2 receptors, activating SMAD and non-SMAD pathways, contributing to immune suppression and the promotion of immune tolerance.
- Interleukin-4 (IL-4) stimulates the differentiation of T cells into Th2 cells, which produce more anti-inflammatory cytokines, promote antibody production by B cells, and inhibit the activation of pro-inflammatory macrophages. It binds to IL-4Rα and IL-13Rα1 receptors, activating the JAK-STAT6 signaling pathway and promoting Th2-type immune responses.
- Interleukin-13 (IL-13) is involved in the regulation of inflammatory responses, particularly in the airways, where it plays a role in allergy and asthma. It binds to IL-13Rα1 and IL-4Rα receptors, activating JAK-STAT signaling pathways, reducing inflammation, and the production of pro-inflammatory cytokines.

These cytokines are essential for maintaining the balance between pro- and anti-inflammatory immune responses, thereby preventing excessive tissue damage and chronic inflammatory diseases. Their dysfunction can lead to various pathologies, such as autoimmune and chronic inflammatory diseases. As a result, they are also potential targets for therapies aimed at modulating inflammation in various diseases [27, 28].

Leukotrienes are another type of signaling molecule involved in the immune response. They are derived from arachidonic acid, which is released from cell membranes when cells are stimulated. Leukotrienes play a role in inflammation, particularly in allergic reactions and asthma. They cause smooth muscle contractions, increase vascular permeability, and attract white blood cells to sites of inflammation. Drugs that inhibit the synthesis or action of leukotrienes are used in the treatment of asthma and other inflammatory conditions.

Prostaglandins are lipid compounds derived from fatty acids. They play a key role in various physiological processes, including inflammation, regulation of blood flow, and modulation of the immune response. These compounds are produced by

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most of the body's cells and act locally in the vicinity of their synthesis. They are involved in the regulation of numerous biological functions, such as the contraction and relaxation of smooth muscles, the modulation of inflammation and immune responses, and the regulation of blood pressure. Prostaglandins are synthesized from arachidonic acid and released by cell membranes in response to various stimuli. The enzyme cyclooxygenase (COX) catalyzes the conversion of arachidonic acid into prostaglandin H2 (PGH2), which is then converted into different types of prostaglandins by specific enzymes. Prostaglandins are involved in many physiological processes and have a variety of effects throughout the body. They are the target of various drugs, including non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit cyclooxygenase activity and consequently reduce prostaglandin synthesis, leading to their anti-inflammatory, analgesic, and antipyretic effects.

1.5.2 Phagocytosis

Phagocytosis is a basic process for nutrition in unicellular organisms, and it is also found in almost all cell types of multicellular organisms. However, only specialized groups of cells called professional phagocytes accomplish phagocytosis with high efficiency. Macrophages, neutrophils, monocytes, dendritic cells, and osteoclasts are among these dedicated cells. Other cells can also interact but at a lower level, such as eosinophils, basophils, and mast cells. This is an important part of the immune response, as professional phagocytes are responsible for eliminating microorganisms and presenting antigens to lymphocytes to initiate an adaptive immune response. Phagocytes can identify several diverse particles that could potentially be ingested, including apoptotic cells and microbial pathogens. Detection of the target particles is mediated by membrane-localized PRRs (see part 1.4) and opsonic receptors. Phagocytosis also helps to eliminate apoptotic cells, debris, and other materials from the body.

1.5.2.1 Phagocytic Cells

Neutrophils and monocytes are produced in hematopoietic tissue and released into the circulation after several stages of determination, proliferation, differentiation, and maturation, which are themselves controlled by various factors.

In the bone marrow, the lineage that will generate blood polynuclear cells, also known as the granulocyte lineage, and the monocyte-macrophage lineage both originate from a common precursor derived from a stem upon granulocyte–monocyte colony-stimulating factor (GM-CSF) stimulation. Cells oriented toward the granulocyte lineage differentiate under the influence of G-CSF into myeloblasts, which mature into promyelocytes, myelocytes, metamyelocytes, non-segmented granulocytes, and neutrophil polynuclear cells. Cells oriented toward the monocyte-macrophage lineage differentiate under the influence of another factor, M-CSF, into monoblasts, promonocytes, and then monocytes.

During these various stages of differentiation, phagocytic cells sequentially acquire the organelles, cytoplasmic constituents, and membrane structures essential for phagocytosis and their other effector functions.

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Neutrophils Granulocytes are named for the densely staining granules in their cytoplasm; they are also called polymorphonuclear leukocytes because of their oddly shaped nuclei. The three types of granulocytes – neutrophils, eosinophils, and basophils – are distinguished by the different staining properties of their granules, which serve distinct functions.

Neutrophils are the most numerous and important cells in innate immune responses. Once they have matured, neutrophils leave the bone marrow and are distributed in the blood in two sectors: one called marginal, where they adhere to the vessel walls; the other called circulating, where they can be counted (from 2 to 7.5 G/l, i.e. 60% or 70% of blood leukocytes). They have a short lifespan (10–12 hours), with adult cells disappearing mainly through destruction in the tissues and intestinal elimination. An increase in the absolute number of neutrophils beyond physiological limits (>8 G/l) is known as granulocytosis. It is caused by two pathophysiological mechanisms: bacterial infection and inflammatory reaction. It can also be induced by certain drugs such as corticoids, which trigger the passage of marrow cells into the circulating blood, or adrenaline, which mobilizes cells of the marginal sector. Conversely, a significant reduction (<0.5 G/l) in neutrophils, or agranulocytosis, may be observed during certain viral infections, drug intoxications, or following particular treatments (chemotherapy and/or radiotherapy) [29].

Neutrophils are characterized as being the first cell line that is recruited at the inflammation site after chemotactic stimuli. These stimuli include complement factors such as C5a factor, chemokines such as IL-8, and leukotrienes (L), including the L-B4, which exerts a paracrine and autocrine function on other neutrophils. They are potent and robust phagocytes. They take up a variety of microorganisms by phagocytosis and efficiently destroy them in intracellular vesicles by using degradative enzymes and other antimicrobial substances stored in their cytoplasmic granules [29, 30].

In addition to phagocytosis, neutrophils exhibit other antimicrobial effector functions:

- **Degranulation:**
  Their cytoplasmic granules play an important role in killing pathogens through degranulation of highly cytotoxic products. They include primary or azurophil granules, which are composed of myeloperoxidase and neutral proteases cathepsin G, elastase, and proteinase 3, as well as defensins. These primary granules are responsible for the release of proteins and peptides that are directed toward pathogen killing and digestion and are the first to be produced during the development of the neutrophil. Secondary or specific granules contain lactoferrin, which sequesters iron and copper. Tertiary or gelatinase granules contain gelatinase proteins such as matrix metalloproteinase-9, which is important for degrading the extracellular matrix and activation of IL-1β, among other functions. Secretory granules contain serum albumin and preformed cytokines. All granules are prevented from release until the engagement of receptors in the plasma membrane or phagosomes. During degranulation or exocytosis, these preformed mediators are released from the granules into the surrounding environment.

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- **NETosis:**
  Upon sensing bacteria in blood, neutrophils can release their DNA in a netlike configuration to create traps known as neutrophil extracellular traps (NETs). NETs are covered with neutrophil proteases (such as elastase), antimicrobial molecules (like histones), and other toxic molecules that kill pathogens. Trapping and killing of pathogens appears to be the primary function of NETs [30].

Monocytes and Macrophages Monocytes are a bone marrow-derived population that make up about 5–10% of white blood cells. In the blood, an increase in the number of monocytes beyond physiological limits generally indicates an active immune response to infection or inflammation. Monocytes remain in the blood for two or three days and then migrate into tissues.

In the tissues, monocytes differentiate into macrophages and, depending on their location, can further differentiate into specialized subsets: Kupffer cells in the liver, alveolar macrophages in the lungs, Langerhans cells in subcutaneous tissue, histiocytes in connective tissue, microglia in the nervous system, splenic macrophages (free or resident) in the spleen, and serous macrophages in the pleura and peritoneum. Tissue macrophages have a long average lifespan, lasting up to one or two months. Macrophages in inflammatory reactions die in situ or are transformed into epithelioid cells and multinucleated giant cells [29].

These cells were fundamental to the understanding of the basic principles of the innate immune response and host defense, with their canonical functions including the phagocytosis of microorganisms, the engulfment of apoptotic/dead cells, and the production of inflammatory cytokines. Once activated, macrophages are armed with several mechanisms to negatively regulate the innate immune response. They can notably trigger anti-inflammatory pathways and facilitate the phagocytosis of apoptotic cells, events required to reestablish tissue homeostasis.

According to this dual function, activated macrophages can be divided into two polarization phenotypes, M1 macrophages and M2 macrophages. M1 macrophages are the most classically known macrophages and are induced by LPS and IFNγ. They secrete pro-inflammatory cytokines such as TNFα, IL-1, or IL-6 and are involved in the elimination of microorganisms and abnormal cells through phagocytosis. Conversely, M2 macrophages are involved in tissue repair and immune tolerance. M2 macrophages are induced by cytokines such as IL-4 and IL-13. They secrete anti-inflammatory cytokines, including IL-10 or TGFβ, and are also potent phagocytes, which act by scavenging debris and inducing both wound healing and angiogenesis [31, 32].

Dendritic Cells (DCs) DCs are considered professional antigen-presenting cells (APC). They reside in and patrol the skin and mucosal surfaces where they detect homeostatic imbalances and process antigens for presentation to T cells, establishing a link between innate and adaptive immune responses (see part 1.6). Antigen uptake occurs through different mechanisms, including phagocytosis, endocytosis, and pinocytosis. Additionally, DCs participate in the induction of peripheral immunological tolerance, regulate the types of T cell immune responses, and function as effector cells in innate immunity against several microbial pathogens [29].

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There are three major subsets of DCs in humans. Common DC progenitors are restricted to the bone marrow, where they give rise to plasmacytoid DCs (pDCs) and to a circulating precursor common to both subsets of conventional DCs (cDCs) found in peripheral lymphoid organs. pDCs can be identified by the expression of CD303. They are specialized to produce type I IFN upon TLR7/9 stimulation and are extremely important in viral infections. The other two subsets are cDC1 and cDC2. cDC1 are characterized by the expression of CD141. They are thought to induce Th1 responses and to be specialized in cross-presentation and $CD8^+$ T cell priming for tumors and pathogens that do not directly infect DCs. cDC2 are characterized by the expression of CD1c. They induce $CD4^+$ T cell priming and promote Th17 and Th2-biased immune responses to extracellular pathogens [33].

1.5.2.2 Process of Phagocytosis

The phagocytic process is mediated by the cytoskeleton of the phagocytic cells as well as by endocytic and signaling receptors [34]. Phagocytosis can be divided into 5 steps:

1) **Attracting the phagocyte**
   After recognizing the pathogen, tissue immune cells release cytokines. The aim of these proteins is to use chemotaxis to attract phagocytic cells circulating in the bloodstream, mainly monocytes, which will then transform into tissue macrophages at the site of infection. Of note, chemotaxis is the ability of certain circulating cells to move under the effect of molecules that attract or repel them.
2) **Adhesion of the pathogenic microorganism to the surface of the phagocyte**
   The pathogen can bind itself directly to the surface of the immune cell via membrane receptors or following opsonization and ligation to the respective opsonic receptor, such as Fcγ receptors, C-type lectins, or complement receptors. This attachment leads to the invagination of the phagocyte's plasma membrane, which can then engulf the microorganism. Invagination is the deformation of the cytoplasm and plasma membrane with the formation of extensions around the target, resulting in a hollow. This is mediated by a complex pathway of membrane proteins and intracellular signaling cascades associated with cytoskeletal rearrangements.
3) **Ingestion of the microorganism by endocytosis**
   The macrophage's plasma membrane deforms, creating pseudopodia that close around the microorganism, leading to its internalization. This is known as endocytosis. Endocytosis consists of the absorption of an element of varying size by a eukaryotic cell, thanks to the invagination of its cytoplasmic membrane.
4) **Formation of a phagolysosome**
   The microorganism is now enclosed in an intracytoplasmic vesicle: the phagosome. The phagosome is surrounded by another type of vesicle present in the cytoplasm of the phagocyte and containing digestive enzymes: the lysosomes. These merge with the phagosome to form a phagolysosome in which the pathogenic microorganism can be digested.

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5) **Digestion of the microorganism**
The phagolysosome is the fundamental microbicidal organelle, equipped with sophisticated mechanisms for degrading microorganisms. First, phagolysosomes are very acidic (pH as low as 4.5) due to the accumulation of many proton pumps called V-ATPases on their membrane. The phagolysosome membrane also presents the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, which is responsible for producing oxygen radicals and their reaction products. This mechanism is known as oxidative burst and its products as reactive oxygen species (ROS). ROS (including O_2^-, $HO^\bullet$, and H_2O_2) are pumped into the phagocytic vacuole, where they contribute to the killing of the microbe. In addition, the phagolysosome contains several hydrolytic enzymes, such as cathepsins, proteases, lysozymes, and lipases, which contribute to degrading ingested microorganisms. The fragments produced by the digestion of the microorganism are expelled from the cell by exocytosis. However, some of the pathogen's protein fragments will be exposed on the phagocyte membrane to activate the adaptive immune response [35].

1.5.3 Pro-coagulant Effects

Inflammation and coagulation are two main host-defense systems that interact with each other, with inflammation activating coagulation and coagulation modulating the inflammatory activity.

Inflammation can trigger the initiation phase of coagulation. Pro-inflammatory cytokines such as IL-6 and TNFα can induce endothelial perturbation, resulting in the release of microparticles, apoptosis, detachment of endothelial cells, and loss of barrier function. In this context, coagulation is activated by the induction of tissue factor on monocytes and endothelium and by the release of von Willebrand factor, which adds to platelet adhesion to the subendothelial surface. Once activated, platelets in turn release pro-coagulant molecules, which promote platelet aggregation and initiate the coagulation cascade. The coagulation cascade results in thrombin formation, which converts fibrinogen into fibrin, thereby promoting clot formation. Some inflammatory mediators can also promote coagulation by inhibiting normal anticoagulant mechanisms, such as protein C, or increasing platelet count and reactivity [36].

On the other hand, inflammatory cells, such as macrophages and lymphocytes, can express receptors for coagulation factors. These interactions between inflammatory cells and coagulation factors can modulate the inflammatory response by promoting the production of inflammatory mediators or by activating immune cells [37].

Activation of coagulation can be considered instrumental in containing inflammatory activity to the site of injury or infection. Indeed, networks of fibrillar proteins, such as fibrin, can form in the affected tissues. These networks can trap pathogens and cellular debris, helping to limit the spread of infection. Coagulation also plays an important role in tissue regeneration. Clot formation can provide structural support for migrating cells during wound healing, and certain

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coagulation factors can stimulate cell proliferation and differentiation. However, inflammation-induced coagulation may also importantly contribute to disease. Excessive activation of coagulation can trigger the formation of microthrombi that can lead to tissue ischemia and damage, or even to systemic activation of coagulation, as illustrated by severe coagulopathies associated with sepsis [37].

1.5.4 Natural Killer Cells

Natural killer (NK) cells are a type of cytotoxic lymphocyte that plays an important role in the innate immune response. Consistent with their function as innate sentinels, NK cells are widespread throughout lymphoid and non-lymphoid tissues. In most tissues, NK cells represent a minor fraction of total lymphocytes. Distinct NK cell subsets have been defined based on phenotypic, functional, and anatomical features. In humans, NK cells can be divided into $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets, which differ in their homing properties. Downregulation of $CD56^{bright}$ expression to become $CD56^{dim}$ and the expression of immunoglobulin superfamily member CD16 (FcγRIII) in a subset of NK cells define stages of maturation. $CD56^{bright}$ NK cells are considered less mature and reside primarily in secondary lymphoid tissues (lymph nodes and tonsils), while around 90% of peripheral blood and spleen NK cells are $CD56^{dim}CD16^{+}$ and express effector molecules as perforin. These $CD56^{dim}$ NK cells are cytotoxic and produce IFNγ upon stimulation in vitro. The intensity and the quality of NK cell cytotoxic and cytokine responses depend on the cytokine microenvironment, as well as on interactions with other cells of the immune system. Type I IFNs, IL-12, IL-18, and IL-15 are potent activators of NK cell effector function. It is also well known that IL-2 promotes NK cell proliferation, cytotoxicity, and, to some extent, cytokine secretion [38].

Of note, naive NK cells do not acquire effector function unless a priming step has occurred by bidirectional interactions between NK cells and activated DCs/macrophages. Upon PRR stimulation, DCs and macrophages generate notably type I IFNs, IL-12, IL-15, and IL-18, which in turn stimulate NK cells to prime. Primed NK cells express CD69 on the cell surface and accumulate intracellular granzyme B but do not spontaneously produce IFN-γ or mediate cytotoxicity [39]. Full activation of NK cells requires additional signals.

NK cells recognize their target cells via a system of germline-encoded activating and inhibitory receptors that recognize membrane-bound ligands on potential target cells (Figure 1.3). Activating receptors include the Fc receptors CD16 and CD32, which efficiently trigger cytotoxicity and cytokine release and enable NK cells to kill antibody-coated cells via antibody-dependent cellular cytotoxicity (ADCC). Other activating receptors expressed by NK cells include C-type lectins (such as NKG2D and NK1.1), natural cytotoxicity receptors (such as NKp46 in mice and humans, and NKp44 and NKp30 in humans), and a few KIRs receptors. Most of the KIR receptors expressed by NK cells are conversely inhibitory receptors. They recognize MHC class I molecules on target cells, and MHC class I loss results in increased NK cell-mediated killing. In addition, humans express a CD94–NKG2A heterodimeric inhibitory receptor, which recognizes peptides derived from classical

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MHC class I molecules, presented by the non-classical HLA-E molecule. As NK cells express different combinations of activating and inhibitory receptors for MHC class I, a selective process is in place to ensure that each NK cell receives sufficient inhibitory signals to counterbalance activation signals and remains tolerant to self. This "fine-tuning" process has been termed education [40].

NK cells exert their cytotoxicity in different ways. Activated NK cells release cytotoxic granules containing proteins such as perforin and granzyme proteases. Perforin binds to the membrane of the target cell and forms pores, facilitating the entry of granzymes. Granzymes are enzymes that activate apoptosis signaling pathways within the target cell. This leads to programmed cell death in the target cell, limiting the spread of viral infection or tumor growth. As mentioned, NK cells also mediate antibody-driven cytotoxicity through ADCC. In addition to their direct cytotoxic activity, activated NK cells secrete a wide variety of cytokines such as IFNγ, TNF-α, GM-CSF, IL-10, IL-5, IL-13, and chemokines such as MIP-1α, MIP-1β, IL-8, and RANTES, which together contribute to the recruitment of inflammatory cells and the maintenance of the effector immune response. IFNγ in particular is one of the most potent effector cytokines secreted by NK cells. IFNγ notably plays a crucial role in antiviral, antibacterial, and antitumor activity by contributing to the activation of macrophages and cytotoxic T cells [21].

1.5.5 Other Innate Lymphoid Cells (ILCs) and Innate-like T cells (ILTCs)

Despite the functional and developmental segregation of many innate and adaptive immune cells, there are cells that cross these frontiers. NK cells, typically, are lymphocytes derived from common lymphoid progenitors, but they are innate immune cells that recognize pathogens using germline-encoded receptors, and they exist in a primed state. Although the importance of NK cells in innate immunity has been known for many years, some other heterogeneous cell populations of innate lymphoid cells (ILCs) and innate-like T cells (ILTCs) have more recently emerged as pivotal regulators of inflammation and as frontier cells between innate and adaptive immunity.

ILCs include NK cells, helper ILCs (termed ILC1s, ILC2s, and ILC3s), and lymphoid tissue inducer cells (LTI). They have the highest degree of innateness and thus do not express a TCR. Helper ILCs are largely tissue-resident cells. They sense perturbations within their surrounding microenvironment, such as alarmins, neuronal mediators, and microbial, dietary, or other environmental molecules, and rapidly respond by secreting effector cytokines, making them one of the first responders in orchestrating immunity, inflammation, and barrier homeostasis [41].

ILTCs, or unconventional T cells, are mainly composed of three key subsets: natural killer T (NKT) cells, mucosal-associated invariant T (MAIT) cells, and γδ T cells. They share phenotypical and functional similarities with both conventional T cells and innate immune cells. Although conventional T cells primarily recognize peptide antigens presented by MHC class I and II molecules (see Chapter 2), ILTCs recognize a diverse range of self- and non-self-molecules. This includes lipid ligands for invariant natural killer T (iNKT) cells, riboflavin derivatives for MAIT cells, and

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phosphoantigens associated with butyrophilins for γδ T cell subsets. These are recognized through their semi-invariant TCR (MAIT, iNKT, and some γδ T cell subsets, for example, Vγ9Vδ2$^+$ T cells) or diverse TCR (type II NKT and other γδ T cells) in an MHC-unrestricted manner. MHC class I-like molecules CD1d and MHC class I-related gene protein (MR1) present antigens to NKT cells and MAIT cells, respectively. Beyond TCR-dependent activation, ILTCs can rapidly sense changes in their environment through the expression of various integrins, chemokines, and cytokine receptors, enabling them to promptly mount potent cytokine responses [41, 42].

Subsets of ILCs and ILTCs can be categorized in accordance with major CD4$^+$ helper T cell subsets type 1, type 2, and type 17 (see Chapter 2), sharing similar functional profiles as defined by common transcription factors, effector molecules, and cytokine receptors. Type 1 ILCs and ILTCs express T-box expressed in T cells (T-bet) and secrete high levels of IFNγ and TNFα. Expression of GATA3 represents a hallmark of type 2 subsets. These cells are characterized as IL-4, IL-5, IL-9, IL-13, and amphiregulin (AREG) secreting cells. Type 17 phenotypes are characterized by the expression of retinoic acid receptor-related orphan receptor-γt (RORγt) and the production of IL-17 and IL-22 [43].

Many of these ILCs and ILTCs are distributed to specific tissues and remain tissue-resident, while others, such as NK cells and some γδ T cells, circulate through the bloodstream. They play an important role by producing effector cytokines at barrier tissue sites, but they also modulate the systemic immune response. They notably influence adaptive immunity through complex communication with myeloid or stromal cell networks, through the provision of soluble factors to support regulatory populations, or through direct cell-surface interactions, including the direct presentation of antigens to T cells. Of note, ILCs and ILTCs interactions with adaptive immune cells seem to be tissue- and context-specific [44].

1.6 Bridging Innate and Adaptive Immunity

Signals from the innate immune system are required for the induction of adaptive immune responses. The cellular and molecular basis by which the innate immune system controls and instructs activation of the adaptive immune system relies on the identification of DCs as the cellular link and the discovery of PRRs as the molecular link between the two branches of the immune system.

1.6.1 Maturation and Migration of Dendritic Cells

DCs possess several key characteristics that enable them to function as the cellular bridge between the innate and adaptive immune systems, especially in the context of the activation of naive T cells. DCs are present in tissues in a functionally immature state with a high level of endocytic and phagocytic activity (see part 1.5). DCs sample antigens in their environment, and antigen engulfment (along with PRR activation) triggers a functional transition known as DC maturation, which is crucial for naïve T cell stimulation through antigen presentation [45].

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First, upon activation, DCs upregulate the CC-chemokine receptor 7 (CCR7), which enables them to migrate and enter secondary lymphoid organs. Second, with an increased lysosomal acidification, DC activation is linked to the degradation of antigens into peptides that are loaded onto the MHC and displayed on the cell surface where they can be recognized by the TCR on T cells (see Chapter 2). Third, activated DCs upregulate the expression of MHC and costimulatory molecules, such as CD80 and CD86, which engage CD28 on T cells. Finally, activated DCs secrete cytokines essential for T cell activation and polarization of the T cell response. DC maturation and migration occur simultaneously, and by the time DCs reach the lymph node, their phenotypic switch is complete: they have low endocytic and phagocytic activity but have become APCs [46].

Of note, macrophages and B cells also are capable of effective antigen presentation. Together with DCs, they are considered professional APCs as they constitutively express MHC class II in addition to class I. Other non-professional APCs can contribute to the modulation of T cell response, including endothelial cells, granulocytes, thymic epithelial cells (TECs), and fibroblasts. In contrast to professional APCs, they express MHC class II only under inflammatory conditions. Among all, activated DCs are considered to have the primary role in the initiation of T cell response according to their large distribution within tissues, their higher surface MHC levels, and their less degradative phagolysosomes, favoring MHC presentation over degradation [2, 28].

1.6.2 Pattern Recognition Receptors in the Control of Adaptive Immunity

Importantly, the induction of an adaptive immune response also relies on PRR activation, which provides information to the DCs and indirectly or directly to T and B cells about the context in which the antigen is presented.

First, PRRs are essential to trigger maturation events mentioned above in DCs. TLR signaling upregulates surface MHC and favors the loading of peptides in phagosomes from which TLR signaling occurs. TLRs and CLRs signaling can also favor the presentation of exogenously acquired antigens on MHC class I (i.e. cross presentation, see Chapter 2). PRR signaling on DCs also induces the expression of costimulatory molecules required for T cell activation. PRRs are thus acting as gatekeepers of adaptive immunity, ensuring that the immune system favors responses against infectious microorganisms or tumor cells rather than normal self. This requirement explains why the use of adjuvants (which was called the immunologists' "dirty little secret" by Janeway) is mandatory to elicit adaptive immune responses to protein antigens.

Beyond initiating maturation events in DCs, PRR signaling can shape their maturation depending on the type of danger detected. Indeed, the combination of PRRs activated in response to various challenges can create different molecular patterns influencing DC response, particularly their cytokine secretion profile. Then, during interaction with T cells, the nature of the cytokines released by DCs plays a decisive role in the differentiation of T cells into different T effector subtypes and eventually

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memory T cells. This process is known as polarization of the T cell response (see Chapter 2). This mechanism ensures that the response is adapted to the type of danger detected (infection with intracellular versus extracellular versus multicellular organisms, notably).

Finally, activation of PRRs, especially TLRs, can directly influence the responses of T and B cells. As an example, B cells express multiple TLRs. In the case of T-independent responses, direct stimulation of TLRs on B cells can either lead to direct activation, as in the case of marginal zone B cells, or serve as costimulatory signals to shape antibody responses by follicular B cells. Memory T cells also express TLRs. TLR2, for instance, acts as a costimulatory receptor on T cells, promoting effector function, cytokine production, and memory generation [47].

1.7 Conclusion

Innate immunity is a multilayered and interconnected system that provides a first line of unspecific immune defenses to the organism and paves the way for the development of specific immune defenses (Figure 1.5).

First, the innate immune response is critical for maintaining homeostasis and preventing microbial invasion by rapidly eliminating a wide variety of pathogens. The constitutive branch of innate immunity provides an immediate response relying on anatomical barriers, antimicrobial molecules, and the complement system. The inducible branch involves various strategies of danger recognition and effector mechanisms. PRRs, which exhibit low polymorphism, can identify "non-self" through a limited number of foreign components shared by many microorganisms (PAMPs, such as lipopolysaccharide, mannose, nucleic acids…) and "damaged-self" through host-derived molecules indicative of cellular infection, stress, or transformation (DAMPs). PRR signaling triggers an inflammatory reaction involving humoral components, such as pro-inflammatory cytokines, and cell-mediated components, such as phagocytic cells (monocytes/macrophages and granulocytes), cytotoxic cells (mainly NK cells), and helper cells (other innate and innate-like lymphoid cells). The inflammatory reaction also mobilizes resident cells, such as epithelial or endothelial cells, and hemostasis components such as platelets and coagulation factors. In other cases, it is a "missing self" recognition that triggers NK cell activation, leading to inflammation.

Furthermore, the innate immune response is essential for initiating and shaping the specific adaptive immune response. PRR signaling is involved in the maturation of dendritic cells, which facilitates antigen presentation and potent activation of naïve T cells. The combination of PRRs activated in response to various challenges also influences the cytokine secretion profile, directly driving T cell differentiation and polarization of the adaptive immune response according to the type of danger detected.

Significant advances have been made in the field of innate immunity since the 1990s, recognizing the central importance of these immune mechanisms and transforming core concepts of immunity in several ways. Notably, while the absence of

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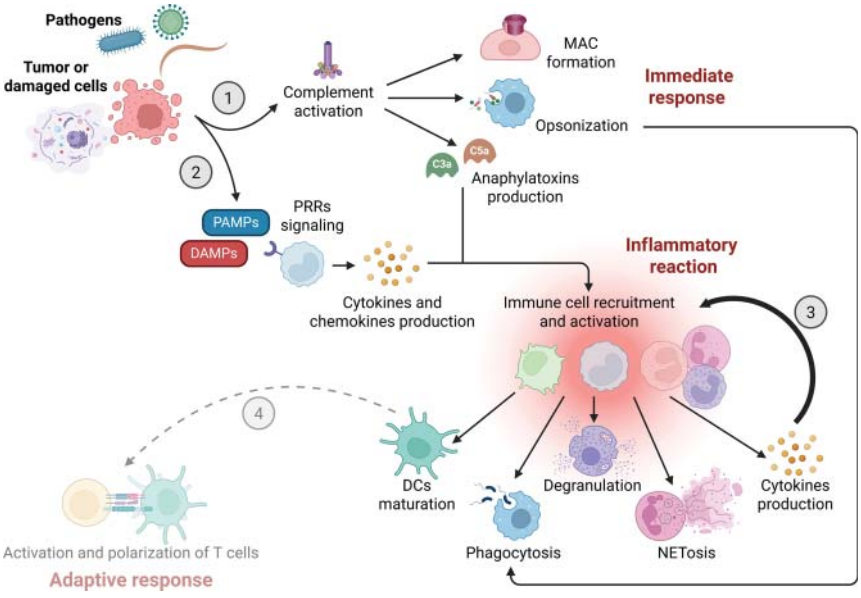

Figure 1.5 Simplified overview of the innate immune response. (1) In the presence of pathogens or abnormal cells, the complement system provides the most rapid response, activating elimination mechanisms such as MAC formation and opsonization, which facilitates phagocytosis, and contributing to innate immune cell recruitment through anaphylatoxins. (2) PRRs can detect PAMPs and DAMPS derived from the initial infection/damage and can trigger the inflammatory reaction by inducing the production of cytokines and chemokines. These molecules attract various innate immune cells to the site of inflammation (granulocytes, monocytes/macrophages, NK cells, and other innate lymphoid cells, DCs) and activate their effector mechanisms. (3) Activated cells will, in turn, produce cytokines at the inflammation site, resulting in a strong amplification signal. (4) PRR signaling and cytokine production also trigger DC maturation, leading to the initiation of the subsequent adaptive immune response through activation and polarization of naïve T cells. Source: "Created with BioRender.com".

immunological memory historically distinguished innate from adaptive immunity, recent work has revealed a collection of innate immune mechanisms that can provide long-term protection, collectively termed "trained immunity" (Section 1.8).

In the specific context of histocompatibility, the innate immune response plays a central role in the pathophysiology of ischemia-reperfusion injury and fetal loss syndrome. It is also involved in initiating allograft rejection mechanisms by triggering adaptive immunity, and more specifically in antibody-mediated rejection through the action of the complement system and ADCC (see Chapter 2). Thus, therapeutic strategies involving complement inhibition are currently being evaluated in transplantation medicine. Innate allorecognition and innate immune memory pathways are also novel targets that will require further and careful investigation in the future.

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1.8 Perspective: The Concept of Trained Immunity

Memory is a central feature of adaptive immunity, relying on clonal selection, somatic recombination, and the persistence of long-lived memory T and B cells, as well as plasma cells (see Chapter 2). However, an immune memory function has also been reported in plants and invertebrates, which lack an adaptive immune system. Since then, a growing body of literature indicates that innate immune cells can display adaptive characteristics and mediate a form of reactivation-based memory, a property that has been termed "trained immunity." As defined by Netea and colleagues, "the concept of trained immunity describes the long-term functional reprogramming of innate immune cells, which is evoked by exogenous or endogenous insults and which leads to an altered response toward a second challenge after the return to a non-activated state. The secondary response to the subsequent non-specific stimulus can be altered in such a way that the cells respond more or less strongly than to the primary response, conferring context-adjusted and time-adjusted responses" [48].

The cellular basis of the protection induced by trained immunity resides in the functional reprogramming of myeloid cells. PAMPs and DAMPs are classic inducers of such training. They can generate trained immunity in the circulation and tissues (that is, peripheral trained immunity) and in HSCs in the bone marrow (that is, central trained immunity). Monocytes derived from trained HSCs migrate to peripheral organs, where they give rise to monocyte-derived macrophages with enhanced effector functions. Of note, an inflammatory memory behavior has also been highlighted in NK cells, ILCs, as well as stromal and epithelial stem cells. Also, the discovery that HSCs and epithelial stem cells display a memory function could explain how short-lived immune cells such as monocytes can acquire memory.

The correct initiation of the mechanisms necessary for the induction of trained immunity relies on the active interplay between epigenetic and metabolic reprogramming of the innate immune cells upon stimulation. Specifically, non-permanent histone modifications associated with gene activation, including H3 Lys4 methylation (H3K4me) and trimethylation (H3K4me3), or H3 Lys27 acetylation (H3K27ac), have been observed in trained macrophages. These epigenetic marks lead to chromatin opening at promoters of genes encoding pro-inflammatory cytokines such as IL-6, IL-1β, and TNFα and are associated with protection against re-infection. Other mechanisms such as DNA methylation or modulation of microRNA and/or long noncoding RNA expression are also expected to be involved. Epigenetic reprogramming is associated with a metabolic shift in trained macrophages, particularly an increase in aerobic glycolysis mediated through a pathway involving the activity of AKT, mechanistic target of rapamycin (mTOR), and hypoxia-inducible factor 1α (HIF1α). The upregulation of glycolysis in trained monocytes is associated with both oxidative phosphorylation and the accumulation of lactate. This metabolic adaptation enhances the capacity of innate immune cells

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to respond to stimulation. Also, certain metabolites derived from these processes, such as fumarate and acetyl coenzyme A (acetyl-CoA), can activate or inhibit a series of enzymes involved in remodeling the epigenetic landscape of cells, such as the histone demethylase lysine-specific demethylase 5 (KDM5) or histone acetyltransferases [49].

Trained immunity programs have evolved as adaptive states that enhance the fitness of the host against secondary immune stimulation (e.g. protective effects after infection or vaccination, or induction of mucosal tolerance toward colonizing microorganisms). Some studies support the hypothesis that trained immunity is one of the main immunological processes that mediate the nonspecific protective effects induced by live vaccines against infections other than the target disease or even against tumors (e.g. bacillus Calmette–Guérin vaccine). However, when inappropriately activated, trained immunity programs can become detrimental, as it has been involved in mechanisms of chronic hyperinflammation (inflammatory diseases, autoimmunity) or a persistent state of immunological tolerance (LPS tolerance and post-sepsis immune paralysis). Of note, in the context of transplantation, sterile inflammation owing to ischemia–reperfusion injury can induce trained immunity and precipitate allograft rejection. Trained immunity is generally reversible and shorter-lived than classical epitope-specific adaptive immunological memory, with phenomena proven to last from 3 months to 1 year (and up to 5 years for heterologous protection against infections induced by live vaccines). However, recent studies have also suggested transgenerational effects through the induction of trained immunity [50].

Abbreviations

ADCC	antibody-dependent cellular cytotoxicity
AKT	a serine/threonine kinase also known as protein kinase B
AMP	adenosine monophosphate
APC	antigen presenting cell
AREG	amphiregulin
ASC	apoptosis-associated speck-like protein containing a CARD
ATP	adenosine triphosphate
C4BP	C4b-binding protein
CARD	caspase recruitment domain
CCR7	CC motif chemokine receptor 7
cGAS	cyclic guanosine adenosine synthase
cGAMP	cyclic guanosine adenosine monophosphate
CD	cluster of differentiation
cDC	conventional dendritic cell
CLR	C-type lectin receptor
COX	cyclooxygenase
CP	classical pathway
CpG	cytosine phosphate guanine

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CR1	complement receptor type 1
CRD	carbohydrate-recognition domain
CS	complement system
DAF	decay-accelerating factor
DAMP	damage-associated molecular pattern
DC	dendritic cell
DIC	disseminated intravascular coagulation
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
DVT	deep vein thrombosis
FcγRIII	receptor III for Fc region of Immunoglobulins
GATA3	trans-acting T-cell-specific transcription factor 3
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte–monocyte colony-stimulating factor
GMP	guanosine monophosphate
GSDMD	gasdermin D
GTP	guanosine triphosphate
HDs	human defensins
HDB1	human β-defensin 1
HIF1α	hypoxia-inducible factor 1 α
HIP/PAP	hepatocarcinoma-intestine-pancreas or pancreatitis-associated protein
HMGB1	high mobility group box 1
HNP	human neutrophil peptide
HSC	hematopoietic stem cell
ILC	innate lymphoid cell
iE-DAP	γ-glutamyl diamino pimelic acid
IFNα/β/γ	interferon α, β, or γ
IgM/G1/G3	immunoglobulin M or G1 or G3
IL	interleukin
IL-4Rα	ınterleukin-4 receptor α
IL-10R1/2	interleukin-10 receptor 1 or 2
IL-13Rα1	interleukin-13 receptor α
ILTC	innate-like T cell
IRF	interferon regulatory factor
JAK1	janus kinase 1
KDM5	lysine-specific demethylase 5
KIR	killer cell immunoglobulin-like receptor
LP	lectin pathway
LPS	lipopolysaccharide
LTI	lymphoid tissue inducer cell
MAC	membrane attack complex
MAIT	mucosal-associated invariant T cell
MAp 19	MBL-associated protein 19
MASP-1/2	mannan-binding lectin serine protease 1 or 2

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MAVS	myeloid anti-viral signaling protein
MBL	mannose-binding lectin
MCP	membrane cofactor protein
M-CSF	monocyte-macrophage colony-stimulating factor
MD2	myeloid differentiation factor 2
MDA5	melanoma differentiation-associated protein 5
MDP	muramyl dipeptide
MHC	major histocompatibility complex
MIP1-α/β	macrophage inflammatory protein 1α or β
MR	mannose receptor
mTOR	mammalian target of rapamycin
MyD88	myeloid differentiation factor 88
NADPH	nicotinamide adenine dinucleotide phosphate
NBD	nucleotide-binding domain
NET	neutrophil extracellular trap
NFκB	nuclear factor kappa B
NK	natural killer cells
NKG2D	natural killer group 2 member D
NKp46	NK protein 46
NKT	natural killer T cells
NLR	NOD-like receptor
NO	nitric oxide
NOD	nucleotide oligomerization domain
NSAID	non-steroidal anti-inflammatory drugs
PAMP	pathogen-associated molecular patterns
pDC	plasmacytoid dendritic cell
PGH2	prostaglandin H2
PRR	pattern recognition receptor
PYD	pyrin domain
RANTES	regulated on activation, normal T cells expressed and secreted
RCA	regulators of complement activation
RIG-I	retinoic acid-inducible gene I
RIP2	CARD-containing serine-threonine kinase
RLR	RIG-like receptor
RNA	ribonucleic acid
ROS	reactive oxygen species
SIRPα	signal regulatory protein α
SMAD	contraction of the *Caenorhabditis elegans* SMA ("small" worm phenotype) proteins and the MAD protein ("Mothers Against Decapentaplegic") in *Drosophila*
ssRNA	single-stranded RNA
STAT3	signal transducer and activator of transcription 3
STING	stimulator of interferon genes
TEC	thymic epithelial cell
TGFβ	tumor growth factor β

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TGFβR	tumor growth factor β receptor
TIR	toll/IL receptor
TLR	toll-like receptor
TNFα/β	tumor necrosis factor α or β
TRIF	TIR domain-containing adaptor-inducing IFNβ

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2

Specific Immune Defence

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2.1 Introduction

The specific immune system, also known as the adaptive immune system, is a highly sophisticated defense mechanism that the body employs to recognize and eliminate specific pathogens. This system operates distinctly from the innate immune system, which provides immediate but nonspecific protection against a broad spectrum of pathogens (Chapter 1). The adaptive immune system, in contrast, generates highly specialized responses tailored to each encountered threat, ensuring targeted and efficient pathogen elimination.

Central for the adaptive immune system is the process of antigen recognition and antigen-specific responses. The detection of foreign antigens – unique molecules present on pathogens – is mediated by specialized receptors on lymphocytes [1]. T cells recognize antigens presented on the surface of infected cells or antigen-presenting cells (APCs) in the context of self-HLA (human leukocyte antigen) molecules. In parallel, B cells produce antibodies that bind to antigens in body fluids or on the surface of pathogens. Upon antigen recognition, these immune cells undergo activation and proliferation, leading to the generation of effector cells that combat the pathogen and memory cells that persist long after the infection is resolved.

A second hallmark feature of the specific immune system is its capacity for generating immunological memory. This feature allows it to "remember" previous encounters with pathogens, enabling faster and more effective responses upon subsequent exposures. Immunological memory is facilitated by various cell types, including T lymphocytes, B lymphocytes, and APCs such as dendritic cells. These cells work in concert through complex signaling pathways and interactions to mount precise immune responses, providing the body with long-term immunity [2].

The specificity and memory of the adaptive immune response provide the host with the ability to mount targeted defenses against a vast array of pathogens, conferring long-lasting immunity. Understanding the details of antigen recognition,

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immune cell activation, and memory formation is fundamental for biomedical technicians. This knowledge is crucial for evaluating, interpreting, and troubleshooting immune-related assays and diagnostics in clinical settings.

The objectives of this chapter are to provide a comprehensive understanding of the specific immune system. It aims to equip the reader with the knowledge needed to grasp the complexities of specific immunity, including its components, functions, and regulatory mechanisms, with a particular emphasis on transplantation. By elucidating the molecular and cellular processes underlying immune responses to both pathogens and transplanted tissues, the chapter will enable readers to accurately interpret and analyze immune-related assays and diagnostics, thereby enhancing their effectiveness in clinical laboratory settings. Understanding these processes is particularly important in the context of transplantation, where immune responses must be carefully managed to prevent graft rejection and ensure transplant success.

2.2 Components of the Adaptive Immune System

2.2.1 T Lymphocytes

T lymphocytes, often referred to simply as T cells, are a critical component of the adaptive immune system, specializing in cellular immunity. These white blood cells are generated in the bone marrow and undergo maturation and selection in the thymus gland. T cells are equipped with T-cell receptors (TCRs) on their surface, which enable them to recognize specific antigens presented by major histocompatibility complex (MHC) molecules on the surface of APCs or infected cells. This TCR-mediated recognition is essential for initiating targeted immune responses against pathogens and abnormal cells, such as those infected by viruses or transformed by cancer.

T cells are versatile and can differentiate into distinct subsets, each with unique roles in immune surveillance and response. Cytotoxic T cells ($CD8^+$ T cells) are primarily responsible for directly killing infected or abnormal cells by releasing cytotoxic molecules like perforin and granzymes, inducing apoptosis in target cells [3]. Helper T cells ($CD4^+$ T cells) play a central role in coordinating and modulating immune responses by releasing cytokines and other signaling molecules. These helper T cells regulate the activity of other immune cells, including B cells, macrophages, and cytotoxic T cells, shaping the overall immune response to effectively combat invading pathogens [4]. Additionally, regulatory T cells (Tregs) maintain immune tolerance by suppressing excessive immune responses and preventing autoimmune reactions, underscoring the crucial role of T lymphocytes in maintaining immune homeostasis and self-tolerance [5].

The diversity and specificity of T-cell responses are fundamental to adaptive immunity, allowing the immune system to tailor its actions to specific threats. Through clonal selection and expansion, T cells generate a diverse repertoire capable of recognizing a vast array of antigens, both foreign and self. This diversity, combined with the ability of memory T cells to persist after initial encounters with pathogens, forms the basis of immunological memory, enabling the immune system to mount faster and more robust responses upon subsequent exposures.

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2.2.2 B Lymphocytes

B lymphocytes, commonly known as B cells, are indispensable components of the adaptive immune system tasked with mediating humoral immune responses through the production of antibodies. Originating from hematopoietic stem cells in the bone marrow, B cells undergo a series of developmental stages that culminate in the acquisition of surface immunoglobulin receptors, which function as antigen receptors. These receptors confer specificity to individual B cells, allowing them to recognize and bind to specific antigens encountered in the body. Upon antigen recognition, B cells become activated, leading to their proliferation and differentiation into plasma cells, the antibody-secreting factories of the immune system [6].

Plasma cells are specialized B cell derivatives dedicated to antibody production, capable of producing large quantities of antibodies tailored to the specific antigens encountered during the immune response. These antibodies, also known as immunoglobulins, circulate in bodily fluids such as blood, lymph, saliva, breast milk, and mucus, where they act as molecular guards, targeting and neutralizing pathogens through various mechanisms. By binding to surface antigens on pathogens, antibodies can prevent their entry into host cells, promote their destruction by immune cells through opsonization or complement activation, or facilitate their clearance from the body. Moreover, B cells possess remarkable plasticity, allowing the antibodies to undergo class switching and affinity maturation, processes that enhance the efficacy and diversity of the antibody response over time [7].

In addition to their role in combating infections, B cells contribute to immune regulation and homeostasis through their interactions with other immune cells and their involvement in the development of immunological memory [8]. Memory B cells, generated during the primary immune response, persist long after the clearance of pathogens, providing the host with rapid and robust protection upon re-exposure to the same antigens. Furthermore, B cells play a crucial role in immune surveillance against cancerous cells, as they can recognize and eliminate tumor-associated antigens, or produce antibodies that mediate antibody-dependent cellular cytotoxicity (ADCC) against cancer cells. Thus, the multifaceted functions of B lymphocytes underscore their importance in adaptive immunity, making them essential targets for therapeutic interventions and diagnostic strategies in the field of biomedicine [9].

2.2.3 Antigens and Antibodies

Antigens and antibodies are fundamental elements of the adaptive immune system, each playing crucial roles in immune recognition, response, and regulation. Antigens are molecules capable of eliciting specific immune responses by binding to antigen receptors on lymphocytes or antibodies. These molecules can be derived from various sources, including pathogens, such as viruses, bacteria, fungi, and parasites, as well as nonpathogenic substances like allergens, toxins, and even components of the body's own cells and tissues. Antigens possess unique molecular

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structures, often characterized by specific regions called epitopes, which can be recognized by adaptive immune cells or antibodies. The immune system's ability to distinguish between self-antigens and non-self-antigens is essential for maintaining immune tolerance and preventing autoimmune reactions.

Antibodies, also known as immunoglobulins (Ig), are specialized proteins produced by B lymphocytes in response to antigen exposure. Structurally, most antibodies basically consist of four polypeptide chains – two heavy chains and two light chains – arranged in a Y-shaped configuration, with the exception of IgM and IgA, which typically form pentameric and dimeric structures, respectively. Each antibody contains antigen-binding sites at the tips of its arms, known as the variable regions, which confer specificity for particular antigens. Through these antigen-binding sites, antibodies can recognize and bind to specific epitopes on antigens with high affinity and specificity. Once bound, antibodies can exert various effector functions to neutralize or eliminate the antigen, including opsonization, complement activation, neutralization, and ADCC.

The dynamic interplay between antigens and antibodies forms the basis of humoral immunity, the arm of the adaptive immune system responsible for combating extracellular pathogens and toxins. Upon encountering antigens, B cells undergo activation and differentiation into plasma cells, which secrete large quantities of antibodies tailored to bind and neutralize the antigen. This antigen-antibody interaction leads to the elimination of pathogens through mechanisms such as agglutination, precipitation, or phagocytosis by immune cells. Additionally, antibodies contribute to the formation of immune complexes, which can activate the complement system and enhance the clearance of pathogens. Moreover, antibodies are crucial for establishing immunological memory, as memory B cells generated during the primary immune response can produce rapid and robust antibody responses upon re-exposure to the same antigen, providing long-lasting protection against recurrent infections. Overall, the coordinated actions of antigens and antibodies are essential for effective immune surveillance, pathogen clearance, and the maintenance of immune homeostasis.

In the context of T cells, antigens play a pivotal role in initiating and shaping cellular immune responses. T cells recognize antigens presented in the context of MHC molecules on the surface of APCs or infected cells. These antigens are typically peptide fragments derived from proteins of pathogens, tumor cells, or other foreign entities. The specificity of T-cell recognition is determined by the interaction between the TCR on the surface of T cells and the antigen-MHC complex. TCRs are highly diverse and capable of recognizing a wide array of antigens, allowing T cells to survey the body for foreign invaders and abnormal cells. Additionally, the affinity of the TCR for the antigen-MHC complex influences the strength and duration of T-cell activation, shaping the magnitude and quality of the ensuing immune response.

The diversity of antigens recognized by T cells is vast, encompassing virtually any foreign protein or peptide presented by MHC molecules. This diversity enables T cells to respond to a broad spectrum of pathogens, including viruses, bacteria, parasites, and fungi, as well as cancer cells and allogeneic cells in the context of transplantation. Moreover, T cells can distinguish between self-antigens and

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non-self-antigens, ensuring that immune responses are directed specifically against foreign invaders while maintaining tolerance to self-tissues. Dysregulation of T-cell responses to self-antigens can lead to autoimmune diseases, whereas inadequate responses to foreign antigens can result in susceptibility to infections or failure to eliminate tumor cells.

2.2.4 Antigen-Presenting Cells

APCs are specialized in the presentation of antigens to T-cells. These cells include dendritic cells, macrophages, and B cells. APCs are essential for initiating and modulating immune responses by processing and presenting antigens on their surface bound to MHC molecules. This antigen presentation is crucial for the activation of T cells and the subsequent adaptive immune response.

Dendritic cells are the most potent APCs, capable of capturing antigens through phagocytosis, endocytosis, and pinocytosis. After antigen uptake, dendritic cells undergo maturation, migrating to lymphoid tissues where they present the processed antigens to naive T cells. This interaction is facilitated by MHC class II molecules on the dendritic cells' surface, which present the antigens to $CD4^+$ helper T cells. Macrophages, another type of APC, also phagocytose pathogens and present antigens to T cells. They play a dual role by both initiating immune responses and by clearing dead cells and pathogens through their phagocytic activity.

B cells, in addition to producing antibodies, can also function as APCs. They capture specific antigens through their B cell receptor (BCR), internalize them, and present processed peptide fragments on MHC class II molecules to helper T cells. This interaction enhances the B cells' ability to produce high-affinity antibodies and supports the formation of memory B cells. The role of B cells as APCs is pivotal in the humoral immune response.

2.3 The Major Histocompatibility Complex and Adaptive Immune Responses

2.3.1 Basics of MHC

The MHC is essential for immune recognition and response via the adaptive immune system [10]. MHC molecules are found in all vertebrates and are referred to as HLA in humans. The MHC molecules play a critical role in presenting peptide antigens to T cells, enabling the immune system to distinguish between self and nonself. MHC/HLA molecules are highly polymorphic, meaning they have numerous variants within the population, as recorded in the IPD-IMGT/HLA database [11]. This high rate of polymorphism allows for a wide range of antigen presentation and immune responses within a species population; the phenotypic diversity is crucial for the immune system's ability to combat a variety of pathogens [12] and plays a significant role in organ transplantation, autoimmune diseases, and vaccine responses.

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2.3.2 Human Leukocyte Antigen (HLA) System

2.3.2.1 General Importance

Given the important role of HLA in immune responses, HLA affects responses to infections, transplantation outcome, disease susceptibility, and tumor control. HLA molecules play a pivotal role in immune surveillance by presenting peptide antigens to T cells, initiating cellular immune responses, and coordinating the activities of other immune cells. This process is essential for distinguishing between self and non-self-antigens, enabling the immune system to mount targeted responses against pathogens while maintaining tolerance to self-tissues. The extensive polymorphism of HLA genes contributes to the diversity of HLA molecules within individuals and populations, allowing for the recognition of a broad spectrum of antigens and enhancing immune responsiveness to diverse environmental challenges. Moreover, the genomic organization of the HLA system reflects evolutionary pressures driving the diversification of HLA genes, including pathogen-mediated selection, reproduction, and genetic drift. This genomic complexity underscores the adaptability and resilience of the immune system, ensuring robust immune surveillance capabilities and the ability to mount effective immune responses against evolving pathogens.

In transplantation medicine, the HLA system plays a critical role in determining the compatibility between donors and recipients of organ and tissue transplants. Matching HLA antigens between donor and recipient is essential for minimizing the risk of graft rejection and optimizing transplant outcomes [13]. Disparities in HLA antigens can trigger alloreactive immune responses, leading to acute or chronic rejection of the transplanted organ or tissue. Therefore, comprehensive HLA typing and matching are important factors for selecting suitable donors and estimating the likelihood of transplant success. Additionally, HLA matching is crucial for minimizing the risk of graft-versus-host disease (GVHD) in the context of allogeneic hematopoietic stem cell transplantation (HSCT), where donor immune cells recognize recipient tissues as foreign and mount immune responses against these host tissues [14].

Furthermore, the HLA system influences disease susceptibility and progression, with specific HLA alleles being associated with increased or decreased risks of various immune-related disorders. For example, certain HLA alleles have been implicated in the pathogenesis of autoimmune diseases, such as HLA-B27 for rheumatoid arthritis [15] and ankylosing spondylitis [16], type 1 diabetes, and multiple sclerosis [17], where they contribute to aberrant immune responses against self-tissues. Conversely, other HLA alleles confer protection against infectious diseases by enhancing immune responsiveness to specific pathogens, for example against SARS-Cov-2 infection [18]. Additionally, HLA diversity plays a crucial role in vaccine-induced immune responses, as individual HLA genotypes can influence vaccine efficacy and the magnitude of immune responses [19]. Therefore, elucidating the role of HLA molecules in disease susceptibility and vaccine responses is essential for developing personalized approaches to disease prevention, treatment, and vaccination strategies.

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2.3.2.2 Structure and Function

The HLA genes encode a diverse array of cell surface proteins crucial for immune recognition and regulation. HLA molecules are divided into two main classes: class I and class II. Class I HLA molecules, composed of HLA-A, HLA-B, and HLA-C genes, are expressed on nearly all nucleated cells and play a central role in presenting endogenous antigens to $CD8^+$ cytotoxic T cells. These antigens typically originate from intracellular pathogens, such as viruses or intracellular bacteria, or from abnormal cells, such as cancer cells. In contrast, HLA class II molecules, consisting of HLA-DR, HLA-DQ, and HLA-DP genes, are predominantly expressed on APCs, including dendritic cells, macrophages, and B cells. HLA class II molecules generally present exogenous antigens acquired from extracellular pathogens, such as bacteria and parasites, to $CD4^+$ helper T cells, initiating immune responses and coordinating the activities of other immune cells. It should be noted that these two pathways are not strictly separated and can be cross presented [20].

The structure of HLA molecules is characterized by a peptide-binding groove formed by α-helices and β-pleated sheets, where antigenic peptides are bound and presented to T cells. The peptide-binding specificity of HLA molecules is determined by polymorphic residues within the peptide-binding groove, allowing for the recognition of a diverse range of antigens. The extensive polymorphism of HLA genes results from genetic variation in the exons encoding the peptide-binding region, leading to the existence of thousands of HLA alleles within the human population. This genetic diversity enables individuals to mount immune responses against a broad spectrum of pathogens and adapt to diverse environmental challenges. Moreover, HLA polymorphism plays a critical role in immune tolerance and transplantation compatibility, as disparities between donor and recipient HLA antigens can trigger alloreactive immune responses, leading to graft rejection or GVHD. Thus, the structure and function of the HLA system are intricately linked, underpinning the specificity and diversity of immune responses and influencing the outcomes of immune-related diseases and transplantation procedures. More detailed information on the structure and the genomic organization of HLA can be found in Chapter 3.

2.3.2.3 Antigen Recognition by the T-Cell Receptor/Interaction

The interaction between the TCR and HLA molecules is highly specific and central to the adaptive immune response. TCRs recognize peptide antigens presented by HLA molecules on the surface of APCs or infected cells. For cytotoxic T cells ($CD8^+$), this interaction occurs with HLA class I molecules, while helper T cells ($CD4^+$) interact with HLA class II molecules. The specificity of this interaction is determined by the complementarity-determining regions (CDRs) of the TCR, particularly CDR1, CDR2, and CDR3, which directly engage with both the antigenic peptide and the HLA molecule.

CDR1 and CDR2, located in the variable regions of the TCR α and β chains, primarily interact with the HLA molecule itself, engaging with the more conserved

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regions of the HLA structure. These regions help the TCR to recognize and bind specifically to the HLA class I or class II molecule. On the other hand, CDR3, which is the most variable region, is mainly responsible for binding to the peptide presented by the HLA molecule. The diversity of CDR3 is generated during T-cell development through V(D)J recombination, allowing the TCR to recognize a vast array of different peptides. This diversity in CDR3 is crucial because it enables the immune system to detect a wide range of pathogens, making the TCR-peptide interaction highly specific to each foreign antigen.

The strength and specificity of the interaction between the TCR and the peptide-HLA complex are influenced by the alignment and fit of CDR1, CDR2, and CDR3 with their respective targets. CDR1 and CDR2 help stabilize the overall interaction with the HLA molecule, while the variable CDR3 ensures specificity for the peptide. This precise recognition process is essential for activating T cells. When the TCR binds strongly to the peptide-HLA complex, intracellular signaling cascades are triggered, leading to T-cell activation, proliferation, and differentiation into effector cells. This interaction is stabilized by the CD4 or CD8 co-receptors, which bind to nonpolymorphic regions of HLA class II or class I molecules, respectively, ensuring the appropriate immune response. The balance between TCR affinity for peptide-HLA and the regulatory influence of CDR1, CDR2, and CDR3 is crucial for immune specificity and tolerance, preventing harmful autoimmunity while ensuring an effective defense against pathogens.

2.3.2.4 Antigen Processing

Antigen processing is the process where proteins are broken down into smaller peptides that are presented on the surface of cells by MHC molecules. These peptides are recognized by T cells, enabling these cells to detect and respond to infections or abnormal cells. There are two main pathways for antigen processing, which correspond to the presentation of peptides by MHC class I and MHC class II molecules.

MHC Class I Antigen Processing MHC class I molecules present peptides derived from intracellular proteins, typically originating from pathogens like viruses or abnormal host proteins produced within the cell (e.g. in cancerous cells). This pathway ensures that cytotoxic $CD8^+$ T cells can monitor and eliminate infected or abnormal cells.

The process begins in the cytoplasm, where proteins are degraded by the proteasome, a complex that breaks down proteins into peptide fragments. These peptides are then transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) proteins. Once inside the ER, peptides are loaded onto newly synthesized MHC class I molecules with the help of chaperone proteins like calnexin and tapasin. After successful peptide binding, the MHC class I-peptide complex is transported through the Golgi apparatus and then to the cell surface.

Once displayed on the cell surface, the peptide-MHC class I complex can be recognized by $CD8^+$ cytotoxic T cells. If the TCR of a $CD8^+$ T cell binds to the peptide presented by the MHC class I molecule, the T cell is activated to kill the infected or abnormal cell, thereby preventing the spread of the pathogen or the growth of abnormal cells.

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MHC Class II Antigen Processing MHC class II molecules generally present peptides derived from extracellular proteins, such as those from bacteria, fungi, and other pathogens that are phagocytosed or endocytosed by specialized APCs like dendritic cells, macrophages, and B cells. This pathway facilitates the activation of $CD4^+$ helper T cells, which are essential for coordinating the immune response by assisting other immune cells, including B cells and cytotoxic T cells.

In the MHC class II pathway, the process begins when an APC engulfs extracellular material through phagocytosis or endocytosis. The engulfed material is contained in a phagosome or endosome, which fuses with a lysosome to form a phagolysosome. Within this compartment, the proteins are broken down into peptides by proteases. Meanwhile, newly synthesized MHC class II molecules are transported from the ER to the endocytic pathway. These MHC molecules are initially bound to an invariant chain, which blocks the peptide-binding groove to prevent premature peptide binding.

In the endosomal compartment, the invariant chain is cleaved, leaving a small fragment called class II-associated invariant chain peptide (CLIP) in the peptide-binding groove. HLA-DM, a specialized molecule, then facilitates the exchange of CLIP for the antigenic peptide in the endosome. Once the peptide is bound to the MHC class II molecule, the complex is transported to the cell surface.

On the surface of the APC, the MHC class II-peptide complex is recognized by $CD4^+$ helper T cells. When a $CD4^+$ T cell's TCR binds to the complex, the T cell becomes activated, initiating an immune response that includes the activation of B cells to produce antibodies and the coordination of cytotoxic T-cell activity.

2.4 T Cell-Mediated Immune Response

2.4.1 T-cell Development

T-cell development is a complex and highly regulated process that begins in the bone marrow, where progenitor cells differentiate into T-cell precursors. These precursors migrate to the thymus, a specialized primary lymphoid organ, where they undergo a series of developmental stages characterized by distinct molecular and cellular changes [21]. During this thymic development, T cells are subjected to rigorous selection processes, including positive and negative selection. Within the thymus, T-cell precursors, known as thymocytes, interact with thymic epithelial cells, dendritic cells, and macrophages, which present self-antigens in the context of MHC molecules. This interaction is crucial for the positive selection of T cells, allowing those with TCRs that moderately recognize self-MHC to survive and mature. Thymocytes that fail to recognize self-MHC undergo apoptosis, a process that eliminates potentially nonfunctional cells. The surviving thymocytes, now referred to as single-positive T cells due to the expression of either CD4 or CD8 co-receptors, proceed to the negative selection phase. Here, thymocytes that bind too strongly to self-antigens are deleted, ensuring that the mature T-cell repertoire is self-tolerant.

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T-cell development involves the differentiation of single-positive thymocytes into either $CD4^+$ helper T cells or $CD8^+$ cytotoxic T cells, depending on the specific interactions with MHC class II or class I molecules, respectively. This differentiation is guided by the strength and duration of signals received through the TCR and co-stimulatory molecules. Once fully developed, mature T cells exit the thymus and enter the peripheral circulation, where they continuously survey for antigens presented by professional APCs. This rigorous developmental process ensures that the T-cell population is both diverse and self-tolerant, capable of mounting effective immune responses while minimizing the risk of autoimmunity.

2.4.2 Activation and Differentiation of T Cells

The activation of T cells requires a precise sequence of interactions between T cells and APCs. T-cell activation begins with the recognition of peptide-MHC complexes on the surface of APCs by the TCR. This recognition is highly specific, with the TCR binding to a unique peptide-MHC combination, ensuring that each T cell responds to a distinct antigen. The initial binding of the TCR to the peptide-MHC complex is insufficient for full T-cell activation; additional signals are necessary. Co-stimulatory molecules, such as CD28 on T cells interacting with CD80 and CD86 molecules on APCs, provide these essential secondary signals. The integration of signals from the TCR and co-stimulatory molecules triggers a cascade of intracellular signaling events, leading to T-cell activation, proliferation, and differentiation.

Once activated, naïve T cells proliferate and differentiate into various effector and memory T-cell subsets, each with specific roles in the immune response. $CD4^+$ helper T cells differentiate into several subsets, including Th1, Th2, Th17, and Treg cells, each characterized by distinct cytokine profiles and functions. Th1 cells produce interferon-gamma (IFN-γ) and are crucial for cellular immunity against intracellular pathogens. Th2 cells secrete interleukins such as IL-4 and IL-5, promoting humoral immunity and responses against extracellular parasites. Th17 cells produce IL-17 and are involved in inflammation and defense against extracellular bacteria and fungi. Regulatory T cells (Tregs), which produce IL-10 and TGF-β, play a vital role in maintaining immune tolerance and preventing autoimmune diseases.

$CD8^+$ cytotoxic T cells differentiate into cytotoxic T lymphocytes (CTLs), which are essential for eliminating infected or malignant cells. CTLs recognize and kill target cells presenting specific antigens in the context of MHC class I molecules. Upon activation, CTLs release cytotoxic granules containing perforin and granzymes, which induce apoptosis in the target cells. Additionally, activated T cells express various surface molecules and secrete cytokines that modulate the immune response, enhancing the activation and function of other immune cells, such as B cells, macrophages, and dendritic cells. The differentiation of T cells into distinct subsets ensures a coordinated and effective immune response, tailored to the nature of the antigen and the type of pathogen encountered.

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2.4.3 Role of Cytotoxic T Lymphocytes (CTLs)

2.4.3.1 Antigen Recognition

CTLs are central to the body's defense against intracellular pathogens and cancer cells; CTLs are able to directly kill infected cells or cancerous cells. The process of antigen recognition by CTLs begins with the presentation of peptide antigens by MHC class I molecules on the surface of target cells. This presentation is vital for CTLs to recognize and respond to intracellular pathogens, such as viruses, and to identify malignant cells that present tumor-associated antigens. The TCR on CTLs binds to the peptide-MHC class I complex with high specificity, initiating the immune response. The co-receptor CD8 on CTLs enhances this interaction by binding to a nonpolymorphic region of the MHC class I molecule, stabilizing the TCR-MHC-peptide complex and ensuring effective antigen recognition.

The recognition of peptide-MHC class I complexes by CTLs is a highly selective process that ensures only cells presenting specific antigens are targeted. This specificity is achieved through the diverse repertoire of TCRs generated during T-cell development. Each CTL expresses a unique TCR that can recognize a particular antigenic peptide. The engagement of the TCR with its cognate antigen-MHC complex triggers intracellular signaling pathways that lead to CTL activation. This activation is enhanced by co-stimulatory signals provided by the interaction of CD28 on CTLs with CD80/CD86 molecules on APCs. These co-stimulatory signals are essential for full CTL activation and function, preventing inappropriate or auto-reactive responses.

Once activated, CTLs undergo clonal expansion, producing a population of effector cells capable of recognizing and killing cells presenting the same antigen-MHC class I complex. This clonal expansion is driven by cytokines such as IL-2, which promotes CTL proliferation and survival. The expanded CTL population circulates throughout the body, surveying for infected or malignant cells. Upon encountering a target cell presenting the specific antigen, CTLs form an immunological synapse, a specialized contact point that facilitates the delivery of cytotoxic molecules. This precise antigen recognition and targeted killing mechanism is critical for the CTLs' role in immune surveillance and the elimination of pathogen-infected or transformed cells.

2.4.3.2 Target Cell Destruction

The destruction of target cells by CTLs is a multi-step process involving the formation of an immunological synapse, secretion of cytotoxic molecules, and induction of apoptosis in the target cell. The immunological synapse is formed at the interface between the CTL and the target cell, allowing for the precise delivery of cytotoxic granules [3]. These granules contain perforin and granzymes, which are crucial for the cytolytic activity of CTLs. Perforin forms pores in the membrane of the target cell, creating channels through which granzymes can enter the cytoplasm [22]. Granzymes are serine proteases that trigger apoptosis by

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activating caspases and cleaving various substrates within the target cell, leading to its programmed cell death.

In addition to granule-mediated killing, CTLs can induce apoptosis through the Fas-FasL pathway [3]. The Fas ligand (FasL) expressed on the surface of activated CTLs binds to the Fas receptor on the target cell. This interaction triggers the extrinsic apoptotic pathway, resulting in the activation of caspase-8 and subsequent caspase cascade, leading to cell death. This pathway is particularly important for eliminating cells that may be resistant to granzyme-mediated apoptosis. The dual mechanisms of granule-mediated and Fas-FasL-mediated killing ensure that CTLs can effectively eliminate a wide range of target cells, including those that have developed mechanisms to evade one pathway.

The ability of CTLs to recognize and destroy target cells is tightly regulated to prevent collateral damage to healthy tissues. CTLs express various inhibitory receptors, such as PD-1 and CTLA-4, which modulate their activity and maintain self-tolerance. These inhibitory signals ensure that CTLs do not become overactivated and cause damage to normal cells. Additionally, the clearance of apoptotic cells by phagocytes helps to limit inflammation and tissue damage. The balance between activating and inhibitory signals, along with the efficient clearance of apoptotic cells, ensures that CTLs effectively eliminate infected or malignant cells while preserving tissue integrity and preventing autoimmune responses.

2.4.3.3 Position in Transplantation (HSCT and Organs)

In the context of transplantation, CTLs play a dual role that can be both beneficial and detrimental, depending on the scenario. In HSCT, CTLs are crucial for the graft-versus-leukemia (GVL) effect, where donor-derived CTLs recognize and kill residual leukemia cells in the recipient, reducing the risk of relapse. This beneficial effect is a key component of the therapeutic success of HSCT in treating hematologic malignancies. However, the same CTLs can also cause GVHD, a condition where donor CTLs attack the recipient's healthy tissues, leading to significant morbidity and mortality. The balance between the GVL effect and GVHD is a major focus of research and clinical management in HSCT, aiming to maximize the therapeutic benefits while minimizing the adverse effects.

In solid organ transplantation, CTLs are primarily involved in mediating acute and chronic rejection of the transplanted organ. Acute rejection occurs when recipient CTLs recognize donor antigens presented by the transplanted organ's cells, leading to an aggressive immune response that can damage or destroy the graft. This process involves direct cytotoxicity by CTLs, as well as the recruitment of other immune cells that contribute to inflammation and tissue damage. Chronic rejection, on the other hand, is a slower, more insidious process that involves ongoing immune-mediated damage and fibrosis, ultimately leading to graft failure. CTLs play a significant role in this process by continually attacking donor antigens and perpetuating an inflammatory environment within the graft.

To mitigate the risk of rejection in organ transplantation, immunosuppressive therapies are employed to inhibit CTL activity and other immune responses. These therapies often include drugs such as calcineurin inhibitors (e.g. cyclosporine,

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tacrolimus), corticosteroids, and antiproliferative agents (e.g. mycophenolate mofetil). While these treatments are effective in preventing acute rejection, they come with significant side effects and increase the risk of infections and malignancies due to the broad suppression of the immune system. Therefore, ongoing research aims to develop more targeted therapies that can selectively inhibit alloreactive CTLs while preserving overall immune function. This includes strategies such as inducing tolerance through regulatory T cells (Tregs), using costimulatory blockade to prevent CTL activation, and developing donor-specific immunosuppressive approaches.

2.4.4 Unconventional T Cells

2.4.4.1 Types of Unconventional T Cells

Unconventional T cells represent a relatively rare subset of T cells, distinct from the conventional $CD4^+$ and $CD8^+$ T cells. These cells exhibit characteristics of both innate and adaptive immune responses and are notable for their lack of MHC restriction. Instead, they recognize monomorphic ligands that are conserved across diverse populations. Key subsets of unconventional T cells include mucosal-associated invariant T (MAIT) cells, natural killer T (NKT) cells, and γδ T cells. MAIT cells, comprising 2–5% of circulating T cells, are predominantly found in tissues such as the liver. They recognize riboflavin-based antigens presented by the nonpolymorphic MR1 molecule, facilitating responses to a broad array of microbial metabolites.

NKT cells are categorized into Type I (invariant NKT or iNKT) and Type II subsets, both recognizing antigens presented by the CD1d molecule. Type I NKT cells, characterized by a highly conserved TCR, respond to lipid antigens like α-galactosylceramide, playing roles in microbial defense and tumor immunity. Type II NKT cells, more prevalent in humans but less understood, exhibit a diverse TCR repertoire and can recognize various lipid antigens, including self and non-self-molecules. These cells are involved in a range of immune responses, from pathogen defense to regulation of autoimmunity.

γδ T cells, which express γδ TCRs instead of the conventional αβ TCRs, constitute approximately 5% of peripheral T cells but are more abundant in tissues. They recognize a variety of antigens, including those presented by butyrophilin molecules and CD1. Vδ2 cells, the most common subset in circulation, respond to phosphoantigens from microbial sources, while Vδ1 cells, more frequent in tissues, recognize lipid antigens presented by CD1 molecules. γδ T cells play critical roles in immune surveillance, particularly in recognizing and responding to stressed or transformed cells, and are implicated in defense against infections, tumor immunity, and autoimmunity.

2.4.4.2 Unconventional T Cells in Transplantation (HSCT and Organs)

Unconventional T cells, such as MAIT, NKT, and γδ T cells, may contribute to both solid organ graft acceptance and rejection [23]. MAIT cells, found in higher frequencies in tissues like the liver, influence transplant outcomes by responding to

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infections and maintaining tissue homeostasis. NKT cells can modulate immune responses post-transplantation, either promoting tolerance or contributing to rejection depending on the encountered antigens. They produce large amounts of cytokines, rapidly influencing the immune environment. γδ are able to respond to stress-induced signals and can contribute to both immune surveillance and tissue repair, which is critical in the context of organ transplantation [24]. As such these cells have been speculated to contribute to allograft tolerance [25].

In HSCT, unconventional T cells are involved in GVHD and GVL effects [26]. MAIT cells can reduce GVHD severity through their regulatory functions and interactions with the intestinal microbiota, with early reconstitution associated with better outcomes. NKT cells influence both GVHD and GVL through cytokine production, balancing immune responses to reduce GVHD while enhancing GVL effects. The γδ T cells contribute to tumor surveillance and GVL by targeting leukemic cells [27], and are important in the defense against viral Infections [28, 29]. On the other hand, they can also cause tissue damage if not properly regulated. These cells' unique roles in transplantation highlight their potential as targets for improving graft outcomes and patient survival through tailored immunomodulatory therapies [30].

2.5 B Cell-Mediated Immune Response

2.5.1 B-Cell Development

B cells, also known as B lymphocytes, are essential components of the adaptive immune system responsible for humoral immunity. The development of B cells begins in the bone marrow, where hematopoietic stem cells differentiate into common lymphoid progenitors. These progenitors give rise to B-cell precursors, which undergo a series of developmental stages characterized by gene rearrangements, selection processes, and the expression of surface immunoglobulins (Ig). The primary goal of B-cell development is to generate a diverse repertoire of BCR capable of recognizing a vast array of antigens while ensuring self-tolerance to prevent autoimmunity.

During the early stages of B-cell development, gene rearrangements occur at the immunoglobulin heavy (IgH) and light (IgL) chain loci. These rearrangements, mediated by the recombination-activating genes (RAG1 and RAG2), create the variable regions of the BCR, providing the basis for antigen specificity. Successful rearrangement of the IgH chain is followed by the rearrangement of the IgL chain, resulting in the expression of a functional BCR on the cell surface. This process is highly selective, with only B cells that successfully rearrange their Ig genes and express a functional BCR progressing to the next stage of development.

The selection of developing B cells involves both positive and negative selection processes. Positive selection ensures that B cells with functional BCRs capable of binding to self-antigens with low affinity receive survival signals. Negative selection

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eliminates B cells that bind strongly to self-antigens, preventing the development of autoreactive B cells that could cause autoimmune diseases. This rigorous selection process occurs primarily in the bone marrow, although additional selection and maturation occur in the peripheral lymphoid organs. Once mature, B cells exit the bone marrow and migrate to secondary lymphoid tissues, such as the spleen and lymph nodes, where they continue to mature and participate in immune responses.

2.5.2 Activation and Differentiation of B Cells

Activation of B cells allows them to respond to specific antigens and produce antibodies. The activation process begins when B cells encounter their cognate antigen, which binds to the BCR on the cell surface. This antigen recognition is enhanced by the co-stimulatory signals provided by helper T cells, particularly through the interaction of CD40 on B cells with CD40 ligand (CD40L) on T cells. Additionally, cytokines produced by helper T cells, such as interleukin-4 (IL-4) and interleukin-21 (IL-21), stimulate B cell activation and proliferation.

Once activated, B cells undergo clonal expansion, producing a population of cells with identical BCRs specific to the antigen. These activated B cells can differentiate into several effector subsets, including plasma cells and memory B cells. Plasma cells are terminally differentiated B cells specialized in antibody production. They secrete large quantities of antibodies that circulate in the blood and lymph, neutralizing pathogens and marking them for destruction by other immune cells. Memory B cells, on the other hand, provide long-term immunity by persisting in the body after the initial immune response has resolved. Upon re-exposure to the same antigen, memory B cells rapidly activate and differentiate into plasma cells, generating a robust and accelerated antibody response.

2.5.3 Role of B Cell Subtypes

2.5.3.1 Naive B Cells

Naive B cells are the resting state of B cells that have not yet encountered their specific antigens. They circulate through the bloodstream and lymphoid tissues, constantly surveying for their specific antigens. Upon encountering an antigen, naive B cells become activated and undergo clonal expansion and differentiation. In the context of organ transplantation, naive B cells can be activated by alloantigens present on the transplanted organ, leading to their differentiation into antibody-secreting plasma cells. This can result in the production of donor-specific antibodies (DSAs), which can mediate antibody-mediated rejection (AMR) and contribute to graft dysfunction. In HSCT, naive B cells may recognize alloantigens on donor hematopoietic stem cells or other graft components, leading to the generation of DSAs and the development of AMR against the transplanted graft. Additionally, naive B cells can participate in the reconstitution of the recipient's B cell repertoire post-transplantation, contributing to the restoration of humoral immunity.

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2.5.3.2 Plasma Cells

Plasma cells are the effector form of B cells, specialized in producing and secreting large quantities of antibodies. Upon activation by antigens and appropriate signals from helper T cells, naive B cells differentiate into plasma cells. Plasma cells produce antibodies that are tailored to bind specific antigens, thereby neutralizing pathogens, promoting opsonization, or triggering other effector mechanisms of the immune system. In the context of transplantation, plasma cells generated post-transplantation may secrete DSAs targeting alloantigens present on the transplanted organ, leading to AMR and graft dysfunction. In HSCT, plasma cells derived from the donor graft may produce antibodies against recipient tissues, resulting in GVHD or other immune-mediated complications. Conversely, plasma cells generated from memory B cells may contribute to protective antibody responses against infectious pathogens in both organ and HSCT recipients, enhancing immune surveillance and host defense mechanisms.

2.5.3.3 Memory B Cells

Memory B cells are long-lived cells generated during the primary immune response to pathogens or vaccination. Unlike plasma cells, memory B cells do not actively secrete antibodies but persist in the body for extended periods, providing immunological memory. Upon re-exposure to the same antigen, memory B cells undergo rapid activation and differentiation into plasma cells, facilitating a more robust and accelerated secondary immune response. In the context of organ transplantation, memory B cells generated during previous encounters with alloantigens may undergo rapid activation upon re-exposure to the same antigens, leading to the production of DSAs and AMR. In HSCT, memory B cells may facilitate the generation of protective antibodies against pathogens encountered by the donor or recipient, contributing to immune reconstitution and infection control post-transplant. The ability of memory B cells to provide long-lasting protection underscores their importance in maintaining immune surveillance and preventing recurrent infections.

2.5.3.4 Regulatory B Cells (Bregs)

Regulatory B cells (Bregs) are a specialized subset of B cells with immunoregulatory functions. Bregs play a crucial role in maintaining immune tolerance and homeostasis by suppressing excessive immune responses and promoting tolerance to self-antigens. They achieve this through the secretion of anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β), as well as by inhibiting the activation and function of effector immune cells. In the context of transplantation, Bregs have the potential to modulate immune responses post-transplantation by suppressing excessive inflammation and promoting immune tolerance. In organ transplantation, Bregs may mitigate alloimmune responses and reduce the risk of rejection by secreting anti-inflammatory cytokines. Similarly, in HSCT, Bregs may play a role in suppressing GVHD and

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promoting tolerance to the transplanted graft, thereby improving transplant outcomes and reducing the need for immunosuppressive therapy. The regulatory functions of Bregs highlight their importance in maintaining immune balance and preventing immune-mediated damage.

2.5.4 Antibody Production

Antibody production is the hallmark of B cell function and a central component of the humoral immune response. Upon activation by antigen and co-stimulatory signals from helper T cells, B cells differentiate into plasma cells, the primary antibody-producing cells. Plasma cells are highly specialized, dedicated to synthesizing and secreting large amounts of antibodies that are specific to the encountered antigen. These antibodies circulate in the bloodstream and lymphatic system, where they perform a variety of functions essential for immune defense.

2.5.4.1 Antibody Classes and Functions

Antibodies, or immunoglobulins (Ig), are classified into five main classes: IgM, IgG, IgA, IgE, and IgD. Each antibody class is adapted to specific roles in the immune system, allowing the body to mount effective responses against a wide range of pathogens and maintain immune homeostasis.

IgM is the first antibody produced in response to an infection and is predominantly found in the bloodstream. It forms pentamers, which allows for efficient binding to antigens and robust activation of the complement system, thereby enhancing pathogen elimination. IgM is essential for the initial defense against pathogens. It is produced by B cells early in the immune response and plays a critical role in agglutination and neutralization of antigens. The BCR for IgM on naive B cells facilitates antigen recognition and subsequent activation of the B cells.

IgG is the most abundant antibody in the blood and extracellular fluid, playing a crucial role in the secondary immune response. It exists in several subclasses (IgG1, IgG2, IgG3, IgG4), each with specific functions. IgG is highly effective at neutralizing toxins, opsonizing pathogens for enhanced phagocytosis, and activating the complement system. It can cross the placenta, providing passive immunity to the fetus and newborns. IgG-specific Fc receptors on macrophages, neutrophils, and natural killer (NK) cells bind to IgG-coated pathogens, facilitating their cellular integration and destruction. IgG's ability to mediate ADCC makes it a key player in immune defense.

IgA is the predominant antibody found in mucosal areas, such as the respiratory and gastrointestinal tracts, and is present in secretions like saliva, tears, and breast milk. It primarily exists as a dimer, which enhances its ability to neutralize pathogens at mucosal surfaces. IgA plays a crucial role in protecting mucosal surfaces from infection and maintaining mucosal immunity. Secretory IgA, produced by plasma cells in mucosal tissues, binds to the polymeric Ig receptor (pIgR) on epithelial cells, facilitating its transport across the epithelium to the mucosal surface. This transport process helps prevent the entry of pathogens into the body.

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IgE is involved in allergic responses and protection against parasitic infections. It binds to high-affinity Fcε receptors on mast cells and basophils. Upon antigen binding, IgE triggers these cells to release histamines and other inflammatory mediators, causing inflammation and aiding in the expulsion of parasites from the body. This mechanism also underlies hypersensitivity reactions, leading to allergic symptoms. IgE plays a significant role in type I hypersensitivity reactions, such as allergic rhinitis, asthma, and anaphylaxis.

IgD is less understood compared to other antibody isotypes but is primarily found on the surface of immature B cells, acting as a receptor for antigens. It is involved in B cell activation and development, signaling the readiness of B cells to participate in the immune response. Although present in low concentrations in the blood, IgD plays a role in initiating and regulating immune responses. The BCR complex containing IgD helps in the activation and maturation of B cells upon encountering specific antigens, thereby contributing to the adaptive immune system's versatility.

B cells can change the isotype of the antibodies that they produce via a process called Isotype switching, also known as class switch recombination. Isotype switching is a crucial process in the adaptive immune system that enables B cells to produce antibodies with different effector functions while retaining antigen specificity. This process allows the immune system to tailor the antibody response to different types of pathogens and environmental challenges. Isotype switching occurs after B cells have been activated by antigen and receive additional signals from helper T cells, particularly through the interaction of CD40 on B cells with CD40 ligand (CD40L) on T cells, as well as cytokines such as interleukin-4 (IL-4), interleukin-21 (IL-21), and transforming growth factor-beta (TGF-β). Moreover, the isotype-switching process relies on the cognate interaction between B cells and helper T cells, known as linked recognition. Linked recognition occurs when a B cell presents a processed antigen on its surface MHC class II molecules to a helper T cell whose TCR is specific for that antigen. This interaction ensures that the B cell and T cell are responding to the same antigen, which is crucial for effective immune responses.

The genetic mechanism underlying isotype switching involves the recombination of the constant region genes of the immunoglobulin heavy chain locus. Initially, B cells produce IgM antibodies, which are encoded by the constant region gene Cμ. During isotype switching, activation-induced cytidine deaminase (AID) initiates the process by introducing double-strand breaks in the DNA at specific switch (S) regions located upstream of the constant region genes. These breaks are then repaired by the nonhomologous end joining (NHEJ) pathway, resulting in the recombination of the V(D)J exon with a different constant region gene, such as Cγ, Cα, or Cε, encoding IgG, IgA, or IgE, respectively. This recombination alters the isotype of the antibody produced without changing its antigen specificity.

The choice of isotype depends on the cytokine environment and the nature of the immune response. For example, IL-4 promotes switching to IgE and IgG1, which are important for responses to parasites and allergens. Interferon-gamma (IFN-γ) promotes switching to IgG1 in humans [31], which are effective in opsonization and complement activation, critical for intracellular bacterial and viral infections.

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TGF-β promotes switching to IgA [32], which is essential for mucosal immunity and protecting mucosal surfaces from pathogens. The ability to switch isotypes enhances the versatility and effectiveness of the humoral immune response.

2.5.4.2 Importance in Immune Response

Antibodies play a critical role in the immune response by recognizing and binding to specific antigens, thereby neutralizing pathogens and marking them for destruction by other immune cells. Through mechanisms such as opsonization, antibodies enhance the phagocytosis of pathogens by macrophages and neutrophils. By activating the complement system, antibodies can lead to the lysis of bacterial cells and the formation of membrane attack complexes. In neutralization, antibodies bind to toxins or viruses, preventing them from interacting with host cells. Additionally, antibodies mediate ADCC, where they bind to infected or cancerous cells and recruit NK cells to induce cell death. These diverse functions of antibodies are crucial for effective immune surveillance and the elimination of pathogens.

2.5.4.3 Effector Functions

The effector functions of antibodies are mediated through their constant (Fc) region, which interacts with various immune cells and complement proteins. The Fc region of IgG antibodies can bind to Fc receptors on macrophages, neutrophils, and NK cells, promoting phagocytosis and ADCC. IgE antibodies bind to high-affinity Fc receptors on mast cells and basophils, leading to the release of histamines and other mediators upon antigen binding, which is important for allergic responses and defense against parasites. IgA antibodies can neutralize pathogens at mucosal surfaces and prevent their entry into the body. The ability of antibodies to engage multiple effector mechanisms ensures a robust and versatile immune response against a wide range of pathogens.

2.5.4.4 Position in Transplantation (HSCT and Organs)

Antibodies play a critical role in mediating graft rejection and contributing to transplant success. In organ transplantation, the production of DSAs by plasma cells can lead to AMR, a major cause of graft failure. DSAs bind to alloantigens on the transplanted organ, activating the complement system and recruiting immune cells that damage the graft. Strategies to prevent AMR include desensitization protocols, the use of immunosuppressive drugs, and the monitoring of DSA levels. Recently, imlifidase, an enzyme that cleaves IgG antibodies, has emerged as a novel therapeutic option. By degrading circulating DSAs and removing the Fc effector domain from the antigen-specific Fab domain, imlifidase can reduce the risk of AMR and improve transplant outcomes. This approach offers a targeted strategy to manage DSAs, complementing traditional desensitization protocols and immunosuppressive therapies.

In HSCT, antibodies may contribute to graft rejection. Pre-existing antibodies against donor stem cells, particularly in allogeneic transplants, can lead to graft rejection by recognizing donor antigens and triggering immune responses that destroy the transplanted stem cells. This is particularly concerning in patients with prior sensitization due to blood transfusions, previous transplants, or pregnancies.

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2.6 Receptors of the Specific Immune System

2.6.1 T-Cell Receptors

2.6.1.1 Structure of T-Cell Receptors

TCRs are specialized protein complexes found on the surface of T cells, playing a crucial role in the immune system's ability to recognize and respond to antigens. Each TCR is composed of two different polypeptide chains, typically designated as alpha (α) and beta (β) chains, though some T cells express gamma (γ) and delta (δ) chains instead. These chains are linked by a disulfide bond and extend through the cell membrane, with both extracellular and intracellular regions. The extracellular region includes the variable (V) and constant (C) domains, with the V domain responsible for antigen binding and the C domain contributing to the receptor's structural integrity.

The antigen-binding site of the TCR is formed by the variable regions of the α and β chains (or γ and δ chains), which create a highly specific binding surface capable of recognizing a diverse array of peptide-MHC complexes. This specificity is determined by the unique combination of amino acids in the variable regions, particularly in the CDRs of the TCR, designated as CDR1, CDR2, and CDR3. These CDRs directly engage with both the antigenic peptide and the HLA molecule. CDR1 and CDR2, located in the variable regions of the TCR α and β chains, primarily interact with the HLA molecule itself, engaging with the more conserved regions of the HLA structure. These regions help the TCR to recognize and bind specifically to the HLA class I or class II molecule. On the other hand, CDR3, which is the most variable region, is mainly responsible for binding to the peptide presented by the HLA molecule. The diversity of CDR3 is generated during T-cell development through V(D)J recombination, allowing the TCR to recognize a vast array of different peptides. This diversity in CDR3 is crucial because it enables the immune system to detect a wide range of pathogens, making the TCR-peptide interaction highly specific to each foreign antigen.

The strength and specificity of the interaction between the TCR and the peptide-HLA complex are influenced by the alignment and fit of CDR1, CDR2, and CDR3 with their respective targets. CDR1 and CDR2 help stabilize the overall interaction with the HLA molecule, while the variable CDR3 ensures specificity for the peptide. This precise recognition process is essential for activating T cells. When the TCR binds strongly to the peptide-HLA complex, intracellular signaling cascades are triggered, leading to T-cell activation, proliferation, and differentiation into effector cells. This interaction is stabilized by the CD4 or CD8 co-receptors, which bind to nonpolymorphic regions of HLA class II or class I molecules, respectively, ensuring the appropriate immune response. The balance between TCR affinity for peptide-HLA and the regulatory influence of CDR1, CDR2, and CDR3 is crucial for immune specificity and tolerance, preventing harmful autoimmunity while ensuring an effective defense against pathogens.

The intracellular portion of the TCR complex includes several signaling molecules, such as CD3 proteins, which are critical for signal transduction

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following antigen recognition. The CD3 complex consists of several chains (CD3γ, CD3δ, CD3ε, and CD3ζ) that contain immunoreceptor tyrosine-based activation motifs (ITAMs). These motifs become phosphorylated upon TCR engagement with an antigen-MHC complex, initiating a cascade of intracellular signaling events that lead to T-cell activation, proliferation, and differentiation. The intricate structure of the TCR and its associated signaling molecules enables the precise and effective activation of T cells in response to antigen recognition.

2.6.1.2 T Cell Receptor Diversity and Gene Rearrangement

The diversity of TCRs is crucial for the immune system to recognize a vast array of antigens presented by pathogens. This diversity is primarily generated through a process known as V(D)J recombination, which occurs during T-cell development in the thymus [33]. The TCR genes are composed of variable (V), diversity (D), and joining (J) gene segments that each have multiple variants on the genome and are randomly selected and rearranged to form the variable regions of the TCR α and β chains. This somatic recombination results in a highly diverse repertoire of TCRs, each with a unique antigen-binding specificity, enabling the immune system to detect a wide range of pathogens.

During V(D)J recombination, recombination-activating genes (RAG1 and RAG2) play a critical role by introducing double-stranded breaks at specific recombination signal sequences flanking the V, D, and J gene segments. These breaks are repaired by the NHEJ pathway, which brings together the selected segments to form a functional TCR gene. Diversity is introduced by the enzyme terminal deoxynucleotidyl transferase (TdT), which adds or deletes nucleotides at the junctions between the segments, creating even greater variability in the TCR repertoire. This additional diversity is essential for the immune system's ability to recognize and respond to countless different antigens.

The process of generating TCR diversity is tightly regulated to ensure the production of functional TCRs that can effectively recognize self-MHC molecules presenting foreign peptides while avoiding self-reactivity. This regulation occurs through positive and negative selection in the thymus. Positive selection allows the survival of T cells with TCRs that recognize self-MHC molecules weakly, while negative selection eliminates T cells with TCRs that bind too strongly to self-antigens. This process results in a mature T-cell population that is both diverse and self-tolerant, capable of mounting immune responses without causing autoimmunity.

The immense diversity generated by V(D)J recombination is critical for the immune system's ability to mount tailored responses against a wide array of pathogens, viruses, and abnormal cells. Each T-cell clone expresses a unique TCR capable of recognizing specific peptide-MHC complexes on APCs. This diversity ensures that T cells can respond to virtually any pathogen the body encounters, providing a robust defense while maintaining self-tolerance.

TdT, an enzyme unique to lymphoid cells, adds diversity during V(D)J recombination by introducing random nucleotide additions or deletions at the junctions between rearranged V, D, and J gene segments. This process, known as junctional

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diversity, contributes significantly to expanding the TCR repertoire. By introducing random nucleotide changes, TdT enables T cells to generate an even broader array of TCRs, potentially enhancing the recognition capability against novel antigens encountered during an immune response.

Junctional diversity ensures that even T cells expressing the same V, D, and J gene segments can exhibit considerable variability in their antigen-binding regions. This variability is critical for optimizing the immune response by fine-tuning TCR specificity and affinity for antigens, thereby facilitating effective immune surveillance and response.

In the thymus, developing T cells undergo a stringent selection process to ensure the maturation of functional TCRs capable of recognizing foreign antigens presented by self-MHC molecules while avoiding reactivity against self-antigens. This process involves positive selection, where thymocytes expressing TCRs that weakly bind to self-MHC molecules receive survival signals and mature into functional T cells. Conversely, thymocytes that fail to recognize self-MHC molecules undergo apoptosis, preventing the emergence of autoreactive T cells that could contribute to autoimmune diseases.

Negative selection complements positive selection by eliminating thymocytes with TCRs that strongly bind to self-antigens presented by self-MHC molecules. This process, also known as central tolerance induction, is crucial for maintaining immunological tolerance and preventing autoimmune reactions against self-tissues.

Together, V(D)J recombination, junctional diversity, and thymic selection collectively shape the diverse repertoire of TCRs expressed by mature T cells, ensuring a finely tuned immune system capable of robust responses against pathogens while maintaining self-tolerance. These mechanisms highlight the sophistication of adaptive immunity in safeguarding the host from infections and maintaining overall immune homeostasis.

2.6.1.3 T-Cell Receptor Signaling Pathways

Upon antigen recognition, T-cell receptors initiate a series of intracellular signaling events that are critical for T-cell activation, proliferation, and differentiation. This process begins with the engagement of the TCR with a peptide-MHC complex on an APC, leading to the phosphorylation of ITAMs on the CD3 and ζ-chain components of the TCR complex. This phosphorylation is mediated by the Src family kinases Lck and Fyn, which are associated with the co-receptors CD4 or CD8, depending on the T-cell subset [34, 35].

The phosphorylated ITAMs serve as docking sites for the SH2 domains of the protein tyrosine kinase ZAP-70, which is subsequently activated through phosphorylation by Lck. Activated ZAP-70 phosphorylates several downstream adaptor proteins and enzymes, including linker for activation of T cells (LAT) and SLP-76, which nucleate signaling complexes at the plasma membrane. These signaling complexes activate multiple downstream pathways, including the phosphoinositide 3-kinase (PI3K)/Akt pathway, the Ras/MAPK pathway, and the NF-κB pathway, each of which contributes to different aspects of T-cell activation and function.

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One of the critical outcomes of TCR signaling is the activation of the transcription factors nuclear factor of activated T cells (NFAT), activator protein 1 (AP-1), and NF-κB, which cooperate to induce the expression of genes necessary for T-cell activation, proliferation, and differentiation. These genes include cytokines like interleukin-2 (IL-2), which promotes T-cell proliferation, as well as other effector molecules and receptors that enhance T-cell function and survival. The integration of signals from the TCR and co-stimulatory molecules, such as CD28, ensures that T cells are fully activated only in the presence of appropriate antigenic and co-stimulatory signals, preventing inappropriate immune responses and maintaining immune homeostasis.

2.6.2 B Cell Receptors/Antibodies

2.6.2.1 Structure of B Cell Receptors and Antibodies

BCR are membrane-bound immunoglobulins (Ig) found on the surface of B cells, playing a critical role in the recognition of antigens. Each BCR consists of two identical heavy chains and two identical light chains, forming a Y-shaped molecule. The tips of the Y shape contain the variable regions (V regions) of both the heavy and light chains, which are responsible for antigen binding. The constant regions (C regions) of the heavy chains form the Fc region, which anchors the receptor to the B cell membrane and mediates effector functions once an antigen is bound [36, 37].

The antigen-binding site of the BCR is formed by the variable regions of the heavy and light chains, which create a highly specific binding surface capable of recognizing a diverse array of antigens. This specificity is determined by the unique combination of amino acids in the CDRs of the variable regions, which directly interact with the antigen. When a BCR binds to its specific antigen, it triggers B cell activation, leading to proliferation and differentiation into plasma cells that secrete antibodies, or into memory B cells that provide long-term immunity.

The strength and specificity of the interaction between the BCR and its antigen are influenced by the alignment and fit of the CDRs. CDR1 and CDR2 help stabilize the overall interaction with the antigen, while the highly variable CDR3 ensures specificity for the antigen's epitope. This precise antigen recognition process is crucial for activating B cells. When the BCR binds strongly to the antigen, intracellular signaling cascades are triggered, leading to B cell activation, proliferation, and differentiation into plasma cells that secrete antibodies. The interaction is stabilized by co-receptors, such as CD19 and CD21, which enhance the signaling strength and ensure an appropriate immune response. The balance between BCR affinity for the antigen and the regulatory influence of CDR1, CDR2, and CDR3 is essential for maintaining immune specificity and tolerance, preventing autoimmunity while enabling an effective defense against pathogens.

Antibodies, or immunoglobulins, share the same basic structure as BCRs but are secreted into the bloodstream and lymphatic system. The structure of antibodies allows them to circulate freely and bind to antigens, neutralizing pathogens, opsonizing microbes for phagocytosis, and activating the complement system. The Fc region of antibodies determines their class and is involved

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in recruiting other components of the immune system, such as macrophages and NK cells, to eliminate the antigen.

2.6.2.2 B Cell Receptor Diversity and Gene Rearrangement

The diversity of BCRs is essential for the immune system's ability to recognize an immense variety of antigens. BCR diversity is generated through V(D)J recombination, which occurs during B cell development in the bone marrow. The principles of BCR gene rearrangement basically parallels the TCR rearrangement processes, with some additional features [38, 39][PMID: 21854230][PMID: 28215280]. The light chains of BCRs (either kappa or lambda) undergo VJ recombination, involving variable (V) and joining (J) gene segments. In contrast, the heavy chains undergo VDJ recombination, which includes variable (V), diversity (D), and joining (J) gene segments. The BCR genes consist of multiple variable (V), diversity (D), and joining (J) gene segments, which are randomly selected and recombined to form the variable regions of the heavy and light chains. This process creates a vast repertoire of BCRs, each with a unique antigen-binding specificity.

During V(D)J recombination, the recombination-activating genes (RAG1 and RAG2) introduce double-stranded breaks at specific recombination signal sequences adjacent to the V, D, and J gene segments. The broken DNA ends are then rejoined by the NHEJ pathway, resulting in the formation of a functional BCR gene. The enzyme TdT increases diversity by adding or deleting nucleotides at the junctions between V, D, and J segments.

In addition to V(D)J recombination, and in contrast to TCR gene development, diversity is introduced through somatic hypermutation and class switch recombination. Somatic hypermutation occurs in the germinal centers of lymphoid tissues, where B cells undergo rapid proliferation and mutation of their variable regions, leading to increased affinity for the antigen. Class switch recombination changes the constant region of the antibody, allowing the B cell to produce different classes of antibodies with the same antigen specificity, thereby tailoring the immune response to different types of pathogens.

2.7 Immunological Memory

2.7.1 Formation and Significance of Immunological Memory

Immunological memory is a fundamental feature of the adaptive immune system, allowing for a more efficient response upon re-exposure to previously encountered pathogens. This memory is formed during the primary immune response, where naive B and T cells are activated by a novel antigen. After the antigen is cleared, most activated cells die, but a subset differentiates into long-lived memory cells that remain in the body for years or even a lifetime. These memory cells are strategically located in lymphoid tissues and peripheral sites, enabling rapid responses to future infections.

Memory B cells, which are formed with the help of T cells, retain refined specificity for previously encountered antigens. In the event of re-exposure to the same

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antigen, these memory B cells quickly differentiate into plasma cells that produce high-affinity antibodies. This rapid response is much faster and more effective than the primary immune response.

In transplantation, memory B cells generated during prior encounters with donor antigens may be problematic, as they can quickly reactivate upon re-exposure to the same antigens, leading to AMR. Managing this response is critical in transplant settings to prevent graft rejection. The significance of immunological memory also extends to its role in immune surveillance, chronic infections, and response to vaccinations.

2.7.2 Secondary Immune Response

The secondary immune response refers to the immune system's reaction to a second or subsequent encounter with the same pathogen or antigen. Because of the presence of memory B and T cells formed during the primary response, the immune system can react more swiftly and powerfully upon re-exposure. This response is characterized by faster initiation, greater magnitude, and enhanced efficiency compared to the primary immune response.

In a secondary response, memory B cells quickly proliferate and differentiate into plasma cells that produce antibodies at a much higher rate than during the primary response. These antibodies, which are often of the IgG class (or IgA in mucosal tissues), have undergone affinity maturation, allowing them to bind the antigen with high specificity and neutralize it more effectively. This rapid production of high-affinity antibodies helps neutralize the pathogen before it can cause significant harm.

Simultaneously, memory T cells are reactivated much faster than naive T cells. Memory $CD4^+$ T cells (helper T cells) quickly release cytokines to coordinate the immune response, enhancing the function of B cells, cytotoxic T cells, and other immune cells. Memory $CD8^+$ T cells (cytotoxic T cells) are able to rapidly recognize and kill infected cells, reducing the pathogen's ability to replicate. This potent response helps prevent the progression of infection and facilitates the rapid clearance of pathogens from the body.

Unlike the primary immune response, which may take several days to become fully effective, the secondary response is often initiated within hours, leading to faster control of the infection. This rapid and robust immune activity usually prevents the development of clinical disease, explaining why individuals who have been vaccinated or previously infected with a pathogen often do not fall ill upon re-exposure.

The secondary immune response is critical for long-term immunity and is the reason why vaccinations can protect against diseases for extended periods, sometimes for life. The efficiency of this response also explains why infections like measles or chickenpox usually occur only once in a lifetime, as the immune system's memory prevents reinfection from causing disease. However, some pathogens, like influenza, can evade immunological memory through mechanisms like antigenic variation, leading to the need for recurrent vaccinations.

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2.7.3 Immunological Memory in Transplantation and Transfusion

Immunological memory affects solid organ transplantation, HSCT, and platelet transfusion, particularly the rapidness of the response. In solid organ transplantation, the presence of pre-existing memory B and T cells, especially those directed against donor-specific antigens, can lead to rapid graft rejection through processes like early antibody-mediated and/or T cell-mediated rejection. This necessitates careful pre-transplant screening and post-transplant immunosuppression to manage immune responses. For HSCT, immunological memory plays a dual role, both in promoting GVL effects and in contributing to complications like GVHD. Effective management of memory T cells is essential to balance these outcomes, allowing for graft survival while minimizing GVHD risk. In platelet transfusion, repeated exposure to donor antigens can lead to alloimmunization, where memory B cells produce antibodies against transfused platelets, resulting in transfusion refractoriness. Addressing these challenges requires strategies like leukoreduction and HLA-matched platelets to minimize immune memory activation, ensuring successful transfusion outcomes.

2.8 Conclusion/Summary/Future

2.8.1 Summary of Key Concepts

In this chapter, we explored the various aspects of the adaptive immune system, a highly specialized defense mechanism that allows for specific recognition and elimination of pathogens. Key components, such as T cells and B cells, were discussed, highlighting their roles in recognizing antigens, activating immune responses, and establishing long-term immunological memory. T cells primarily mediate cellular immunity, directly targeting infected or abnormal cells, while B cells are central to humoral immunity through antibody production. The concepts of antigen presentation, TCR diversity, and BCR gene rearrangement were fundamental to understanding how the immune system generates specificity and diversity. Additionally, the chapter covered immunological memory, a cornerstone of adaptive immunity, ensuring faster and more effective responses upon subsequent exposures to the same pathogens. This knowledge is essential for applications in transplantation, where immune responses must be carefully managed to prevent graft rejection and ensure successful outcomes.

2.8.2 Importance of Ongoing Research in Immunology

Ongoing research in immunology is key for advancing our understanding of immune mechanisms and for developing innovative treatments for a range of conditions, from infections to cancers. In transplantation medicine, research continues to explore how immunological memory and the immune system's specificity can be better managed to reduce the risk of rejection, particularly in cases involving pre-existing memory responses to donor antigens. Advances in

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therapies that target immune regulation, such as immunosuppressive drugs and antibody-cleaving enzymes, are crucial for improving patient outcomes in both solid organ transplantation and HSCT. Furthermore, the exploration of immune tolerance and the development of personalized medicine based on an individual's immune profile will shape future interventions.

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3

The HLA/MHC System

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3.1 Introduction

The HLA family of genes and molecules are the cornerstone of the field of Histocompatibility and Immunogenetics. Since the discovery of the *HLA* loci in the late 1950s [1], our knowledge of the structural formation of both the HLA molecules and genes that encode them has grown enormously. Our understanding of the function of the HLA molecules and their clinical relevance has grown correspondingly, with the transplantation, drug hypersensitivity, and disease association testing being some of the many clinical applications where HLA is thought to be involved. The extent of diversity of the *HLA* genes is incomparable, causing many challenges to scientists in the Histocompatibility and Immunogenetics field, but enabling scientists and clinicians to provide personalized medicine for their patients long before this was considered by the wider medical field.

This chapter aims to provide a background on the basics of the HLA family of genes and proteins, covering their structure, function, diversity, and the tools that enable us to navigate our way through their enormous complexity. Although there are many *HLA* genes, this chapter will predominantly focus on the genes and characteristics of the classical HLA molecules, HLA-A, -B, -C, -DR, -DQ, and -DP as they are the most frequently studied and tested molecules. More detailed discussion on the nonclassical *HLA* and *HLA*-like loci can be found in Chapter 7.

3.1.1 The Major Histocompatibility Complex

The extended human Major Histocompatibility Complex (MHC) is located on the short arm of chromosome 6 (6p21.3) and, in its entirety, spans a region of approximately 7.6 megabase pairs. This region consists of several hundred expressed genes that predominantly have some function within the responses of the immune system, while over 100 additional pseudogenes have also been identified [2, 3]. It is thought to be one of the most gene-dense regions of the human genome. There are

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three distinct regions of the MHC known as the class I, II, and III regions. The class I region is closest to the telomere, while the class II region is the most centromeric, with the intervening class III region positioned between them. The genes within the class I and II regions include the *HLA* genes, while the genes located in the class III region include components of the complement system and cytokine genes [4, 5].

3.1.2 The *HLA* Gene System

There are currently 45 different *HLA* genes that have been identified across the MHC, including the recently identified *HLA-OLI* [6], which has subsequently been given the official nomenclature *HLA-R* by the World Health Organization (WHO) Nomenclature Committee for Factors of the HLA System [7]. Several of the identified *HLA* genes are non-expressed pseudogenes, although recent evidence for at least one of these genes (*HLA-H*) may be conflicting [8, 9]. The family of HLA genes are frequently referred to as the HLA class I or class II loci depending on which region of the MHC they are located, and their structural and functional characteristics.

Typically, chromosome 6 in humans contains a copy of each of the known *HLA* genes with two important exceptions. First, a large section (approximately 50 kilobase pairs (kb)) of the MHC class I region that notably contains the *HLA-H* gene is deleted in some individuals [10]. This has predominantly been observed in individuals where *HLA-A*23* and *A*24* alleles are present on their MHC haplotypes. Second, there are distinct HLA class II haplotypes that contain specific combinations of the *HLA-DRB* genes. Further details about the *HLA-DR* haplotype structures can be found in section 3.1.5.

3.1.3 HLA Class I Genes and Molecules: Structure and Function

The expressed *HLA* class I genes are co-dominantly inherited and are approximately 3 to 4.5 kb in length. The classical *HLA* class I genes consist of seven or eight functional exons. These genes encode mature proteins of approximately 340 amino acids.

The HLA class I molecules are co-dominantly expressed on the surface of most nucleated cells (Figure 3.1). The encoded protein forms an α-chain protein consisting of three extracellular domains (α1, α2, and α3), which are encoded by exons 2, 3, and 4, respectively, a transmembrane anchor (exon 5), and a cytoplasmic tail (exons 6 and 7, and in the case of *HLA-A* and *-C*, exon 8 as well). Although not part of the mature protein, exon 1 encodes a short leader peptide that is subsequently bound and presented by the nonclassical HLA-E molecule.

In addition to the α-chain, HLA class I molecules also contain a β_2-microglobulin subunit that binds non-covalently to the α-chain, providing stability to the mature protein structure. The gene encoding β_2-microglobulin (*B2M*) is located on chromosome 15q21.1, and although several variants of the gene have been identified, these have predominantly been found in individuals with immunodeficiency or malignant diseases and are possibly mutations that have caused disease, rather than natural variation. Therefore, in healthy individuals, *B2M* is generally considered to be monomorphic.

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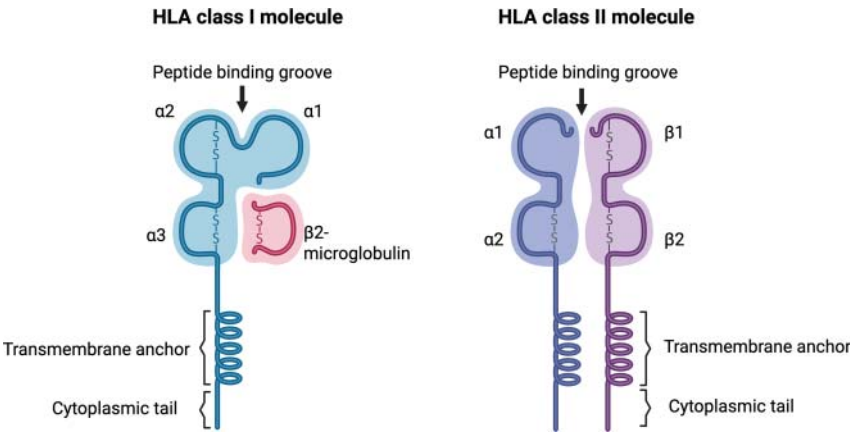

Figure 3.1 Illustration of the structure of the HLA class I and II molecules. Although structurally and functionally similar, there are notable differences between the two molecules. HLA class I molecules are encoded by a single gene creating an a-chain, with the α1 and α2 domains forming the peptide binding groove. The HLA class II molecule is encoded by two separate genes producing an α- and β-chain, with the α1 and β1 domains forming the peptide binding groove of these molecules. Source: Created with BioRender.com.

The α1 and α2 domains of the HLA class I molecule form a peptide-binding groove, also frequently referred to as the antigen recognition domain, in which digested antigenic peptides are bound for presentation to specific T cell receptors [11]. The peptides presented by HLA class I molecules are thought to be on average nine amino acids in length, although evidence suggests they can be significantly longer [12, 13]. The peptides are bound to the HLA molecule via anchor residues at the carboxy terminus and toward the amino terminal end of the peptide [14]. Further details on the HLA class I antigen presentation pathway can be found in Chapter 2.

3.1.4 HLA Class II Genes and Molecules: Structure and Function

The *HLA* class II genes are significantly longer than their class I counterparts, ranging from 8 to 16 kilobase pairs. The divergent lengths of genes occur not only between loci, but also within a single gene. Most commonly observed are the differences in the length of the *HLA-DRB1* allele sequences, caused by large insertions within introns 1 and 2 that are particularly found in sequences of *HLA-DRB1*04, 07,* and *09* alleles. The mature proteins encoded by the class II genes are approximately 230 amino acids in length.

The structure of HLA class II molecules is quite different to HLA class I, with a heterodimer arrangement formed from an α- and a β-chain that are encoded by two distinct genes within the class II region of the MHC, for example, proteins encoded by *HLA-DQA1* and *-DQB1* genes form the HLA-DQ molecule (Figure 3.1). Although differently encoded, the structure of the α- and β-chain proteins is similar. Analogously to the HLA class I genes, individual exons of the HLA class II genes encode distinct sections of the molecules. As with *HLA* class I genes, exon 1 encodes

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a leader peptide. Exons 2 and 3 encode the two extracellular domains of both the α- and β-chains: α1 and α2, and β1 and β2, respectively. The transmembrane domain and cytoplasmic tail are encoded by exons 4 and 5 of the α-chain gene, but only by exon 4 of the β-chain gene.

Both the α1 and β1 extracellular domains form the peptide-binding groove of the HLA class II molecules. The length of an HLA class II peptide can be considerably larger than their class I comparators due to the structural formation of the peptide-binding groove. The open-ended nature of this part of the HLA class II molecules accommodates an extended length, enabling the peptides to protrude at either end, with binding occurring at multiple points throughout the groove [14]. Consequently, the average peptide length is thought to be 15 amino acids but can range from 10 to 34 amino acids. The peptide is "gripped" at its center within the class II molecule by the binding of anchor residues at multiple sites.

HLA class II expression is limited to antigen-presenting cells that are involved in immune responses such as B cells, monocytes, macrophages, and dendritic cells, as well as tissue equivalents, for example, Kupfer cells found in the liver. Further details on the HLA class II antigen presentation pathway can be found in Chapter 2.

3.1.5 The HLA-DR Haplotype Structures

The α-chain of the HLA-DR molecule is encoded by the *HLA-DRA gene*. Once thought to be monomorphic, there are now 17 HLA-DRA proteins identified [15] (Table 3.1). There are nine *HLA-DRB* genes, four of which are expressed (*HLA-DRB1, -DRB3, -DRB4,* and *-DRB5*) and five of which are pseudogenes (*HLA-DRB2, -DRB6, -DRB7, -DRB8,* and *-DRB9*). *HLA-DRB1* is expressed at a higher level than *HLA-DRB3, -DRB4,* or *-DRB5*.

Table 3.1 Number of Unique HLA alleles and Proteins. The number of unique alleles and identified proteins of the classical HLA class I and II loci as of July 2024, Release 3.57.0 of the IPD-IMGT/HLA Database.

	Number of alleles	Number of proteins
HLA class I genes		
HLA-A	8,381	4,902
HLA-B	10,080	6,017
HLA-C	8,454	4,662
HLA class II genes		
HLA-DRA	78	17
HLA-DRB1	3,714	2,405
HLA-DQA1	808	407
HLA-DQB1	2,602	1,566
HLA-DPA1	698	343
HLA-DPB1	2,607	1,503

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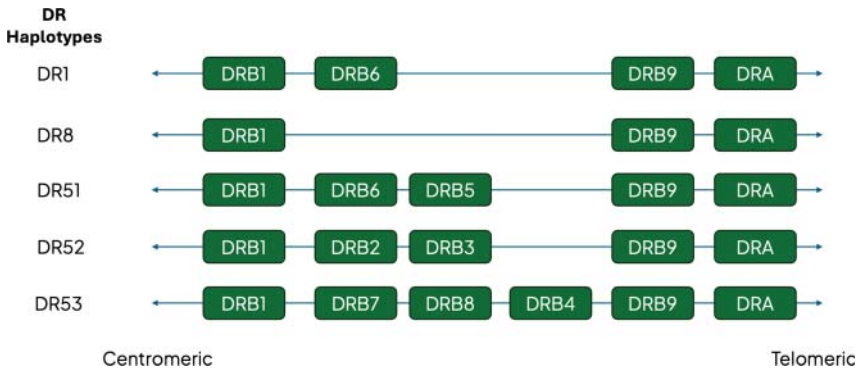

Figure 3.2 Illustration of the genetic composition of the known HLA-DR haplotype structures. There are five known HLA-DR haplotype structures each with unique combinations of the *HLA-DR* genes [16]. All haplotypes contain the expressed *HLA-DRB1* and *-DRA* genes as well as the *HLA-DRB9* pseudogene. Only the HLA-DR51, -DR52, and -DR53 haplotypes contain additional expressed HLA-DR genes (*HLA-DRB5, -DRB3, and -DRB4,* respectively). The distances between genes are not to scale.

Five distinct DR haplotype structures have been determined, known as DR1, DR8, DR51, DR52, and DR53, each of which contains a unique combination of the expressed genes and pseudogenes, named after the defining antigens they contain (Figure 3.2) [16]. The DR1 and DR8 antigens result from products of the *HLA-DRB1* gene, while the DR51, DR52, and DR53 antigens result from products of the *HLA-DRB5, -DRB3,* and *-DRB4* genes respectively. All haplotypes contain the *HLA-DRB1, -DRB9,* and *-DRA* genes. However, no commonly known haplotype contains more than one additional expressed *HLA-DRB* gene and the DR1 and DR8 haplotypes are not known to contain any. The *HLA-DRB3* gene is found on the HLA-DR52 haplotype, which is associated with the *HLA-DRB1*03, 11, 12, 13,* and *14* allele families. The *HLA-DRB4* gene is found on the HLA-DR53 haplotype and is associated with the presence of *HLA-DRB1*04, 07,* and *09* allele families. The HLA-DR51 haplotype contains the *HLA-DRB5* gene and is associated with the *HLA-DRB1*15* and *16* allele families. The remaining *HLA-DRB1*01, 08,* and *10* allele families do not occur on any haplotype expressing the *HLA-DRB3, -DRB4,* or *-DRB5* genes. Any exception to these known HLA-DR haplotype structures is rare, with the observation of *HLA-DRB5* on haplotypes containing *HLA-DRB1*01* alleles being the most frequently reported [17].

3.1.6 HLA Polymorphism

The HLA genes are the most polymorphic genes of the human genome [18], with over 39,600 alleles identified to date (Release 3.57.0; July 2024; www.ebi.ac.uk/imgt/hla) [19, 20]. This increased level of variation is termed hyperpolymorphism [21], and to date, the majority is found within six classical HLA genes: HLA class I genes *HLA-A, -B,* and *-C* and HLA class II genes *HLA-DRB1, -DQB1,* and *-DPB1*. However, the significant transition to next- and third-generation sequencing as HLA typing

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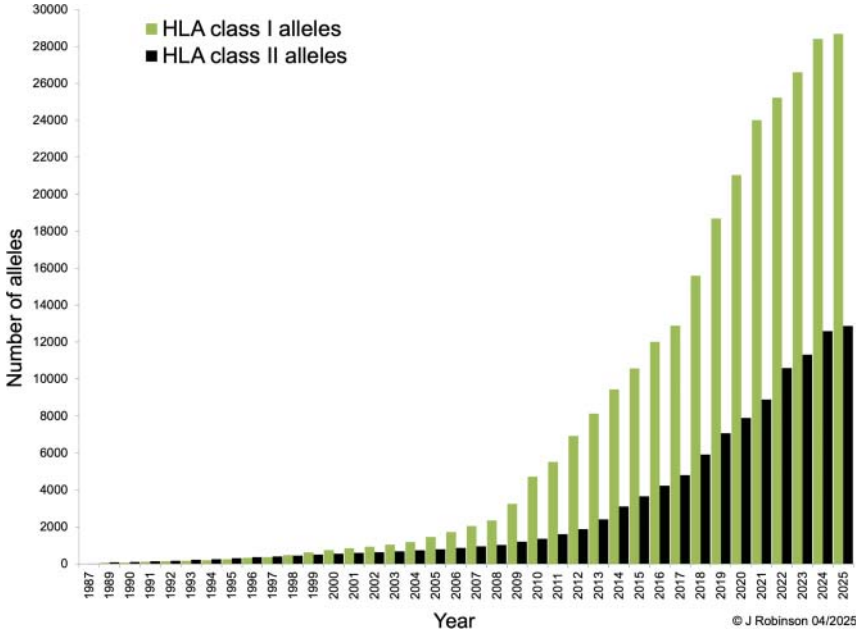

Figure 3.3 The extent of HLA diversity in 2025. The number of unique identified HLA class I and II alleles described increases each year, with the rate of novel allele discovery changing in line with changes in HLA typing technologies. Source: Adapted with permission from James Robinson [19].

methods, coupled with the increased interest in discovering the extent of diversity within the MHC, has notably affected the number of variants observed (Table 3.1 and Figure 3.3) [20, 22]. Recent analyses of exons 2 and 3 of the classical HLA class I genes have suggested that the variation observed within these genes is likely to be an indication of possible numbers, with millions of alleles predicted to exist globally [21]. In order for this degree of variation to be managed and easily communicated, a formal nomenclature system is required. See sections 3.1.9 for further details.

The majority of polymorphism identified to date has been found within the exons encoding the peptide binding grooves and the points of the molecule that interact with T cell receptors. This functional diversity is a mechanism to ensure ongoing pathogen recognition and eradication by enabling an increased repertoire of peptide motifs that can be bound and presented to T cells [23, 24]. Additionally, the extent of HLA variation has likely also been driven by over dominant selection, with a significant benefit associated with HLA heterozygosity in a population, resulting in increasing numbers of possible peptide motifs that can be bound and presented by the co-dominantly expressed HLA molecules [24]. However, as increasing numbers of HLA typing laboratories are expanding the regions of the genes that are sequenced, significant intronic and noncoding variation is also being observed [19, 20].

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3.1.7 How Does Variation Arise within *HLA* Genes?

There are many different mechanisms by which new *HLA* gene sequences arise [21]. The type of novel gene sequences most frequently observed results from the substitution of nucleotides. This can either be simple, with single nucleotides substitutions, often referred to as single nucleotide polymorphisms, occurring at a single point within the gene sequence, or more complex when longer string of nucleotides are changed.

The insertion or deletion of one or more nucleotides within the allele sequence is also frequently observed. While this can occur at any point within the gene sequence, certain areas are more prone to these types of changes. Long stretches of a single nucleotide repeated consecutively (e.g. GGGGGGGG) are known as homopolymer regions, while the repetition of a particular sequence (e.g. ATCATCATCATC) is known as a repeat region. The repetitive nature of both types of variation makes these sections prone for novel variation to arise. However, the complex nature of these regions also makes sequencing and typing of them more challenging, and data should be thoroughly interrogated to ensure novel variation at these sites is genuine.

The final mechanism by which variation has arisen within the *HLA* loci is via recombination events, occurring when the sequences of two distinct alleles are combined to create an entirely new sequence. This often results as either a single recombination event, when the start of one gene is recombined with the end of another, or a double recombination event, where only a small section of a sequence is inserted into another. Within the *HLA* family of genes, recombination events can be complex, with evidence of this happening not only between alleles of the same locus, but also between different genes. An example of single recombination events is *HLA-A*69:01* that is believed to have resulted from a single recombination of the *HLA-A*02:01* and *A*68:01* alleles [25], while *HLA-A*11:335* has likely resulted from a recombination event between an *HLA-A*11* and *HLA-H*02* allele [26].

3.1.8 Haplotypes and Linkage Disequilibrium

The original description of a haplotype was reported by Ruggero Ceppellini, who defined it as the genetic constitution of a single chromosome, either the combinations of genes or the alleles seen at particular genetic loci [27, 28]. Within the HLA field, both definitions of haplotype are used, for example, when describing the HLA-DR haplotypes (see section 3.1.5), or when describing common patterns of inheritance of particular alleles of the *HLA* genes.

These common patterns of allelic inheritance result from extensive linkage disequilibrium that is observed within the MHC region. Linkage disequilibrium is defined as the nonrandom assortment of genetic factors, with the frequency of a haplotype containing two or more alleles being either noticeably higher or lower than is expected at random [29]. Notably, the tight linkage disequilibrium is observed between *HLA-B* and *-C* and between *HLA-DRB1* and *-DQB1* largely driven by their close proximity within the MHC. In addition to this, common HLA haplotypes are also observed, where distinct combinations of alleles are observed across all the classical *HLA* genes. As the MHC and *HLA* loci have

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evolved, particular HLA haplotypes have predominated in different populations. For example, one of the most well-characterized and frequently observed HLA haplotypes in individuals of North European ancestry is the *HLA-A*01:01~C*07:01~ B*08:01~DRB1*03:01~DQB1*02:01* haplotype (also known as the HLA 8.1 haplotype), while in East Asian populations, *HLA-A*24:02~C*12:02~B*52:01~DRB1*15:02~DQB1*06:01* predominates [27, 28].

3.1.9 Nomenclature

The extensive levels of variation and the necessity to accurately perform HLA typing require the use of a common nomenclature to ensure the accurate exchange of information. Without a controlled vocabulary and the accurate assignment of a unique identifier to each unique sequence, it would be near impossible to categorize, identify, and exchange HLA typing information on individuals and compare "HLA types". This concept was recognized in the very early research into HLA and predates DNA sequencing, and as a result, the nomenclature has evolved to support sequencing technologies and increased allele numbers. A dedicated nomenclature for HLA was developed in the late 1960s [30] with the earliest versions focusing on identifying the distinct HLA genes and later naming genes and defining serological variants, the HLA antigens.

These early versions of the nomenclature defined a number of serological definitions, which refer to the antibody reaction to specific HLA antigens [1, 31]. The nomenclature used for HLA serological definitions is based on gene names and local designations at the time of assignment, for example, A1, which refers to the serological specificity associated with the *HLA-A*01* alleles, or B7, which is the *HLA-B*07* alleles. It should be noted that for HLA class II serological definitions, the B in the gene names is not included, for example, DR3 for the *HLA-DRB1*03* alleles. Serological nomenclature may also contain a "w" which indicated a "workshop status"; some of these "w's" remain to help differentiate HLA-C serological definitions, for example, Cw1 and Cw2 are used to differentiate from the complement factors like C1 or C2. Not all HLA genes have defined serological definitions.

As the understanding of HLA antigens grew, largely driven by improvements in the typing methods used for their characterization, it became clear that some recognized antigens comprised of more than one single group. To help differentiate between the groups, the terms "Broad" and "Split" antigens were used. The differentiation of antigens was often simple, for example, the A9 broad antigen was subsequently subdivided into two split antigens, the A23 and A24 antigens. However, in other situations, this differentiation was far more complex. A prime example of this was the broad antigen B15, which was further subdivided into the split antigens B62, B63, B75, B76, and B77.

The earliest nomenclature did not use the letters HLA to refer to the "human leukocyte antigens"; rather, it originally referred to Human Locus-A with the abbreviation HL-A [30]. The name HLA replaced HL-A in 1975 when it was understood that the products of two different genes had been identified. Thus, HL-A became *HLA-A* and *HLA-B* [32]. The use of HLA as a backronym referring to the "human

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leucocyte antigens" is reported to have been coined by scientists working outside the MHC field, and slowly adopted for its more common incorrect usage today.

Once DNA sequences were identified, the nomenclature needed to be expanded to allow for the assignment of unique codes to what are now termed HLA alleles. Within the HLA field, the term "allele" refers to the combination of point mutations, insertions, and deletion that are seen in a single phased sequence for any given gene. Each allele can therefore be made up of multiple variant positions when compared to a single reference sequence per gene. In its simplest form, the nomenclature assigns a unique label to each unique DNA sequence; however, the nomenclature can also inform the reader of more information about the sequence. An allele name can be split into distinct parts: the "HLA-" prefix, followed by the gene designation and an asterisk. Following this, up to four numerical fields are included, each separated by a colon. The colon was introduced in 2010 due to some allelic groups exceeding 99 variants; previous examples of the nomenclature are shown in Figure 3.4. The first field represents the main allele group and is often linked to the serological definition, for example, the majority of the *HLA-A*02* alleles will have the A2 serology. The second field represents a distinct protein sequence, and all allele names that start with the same two fields should have the same translated amino acid sequence. The third field represents silent variations within the coding sequence, i.e. the combined exon sequences. This means any alleles that share the same three fields will have the same coding sequence. Finally, the fourth field allows for identification of variation outside the coding exons, including those in introns or the 5′ or 3′ untranslated regions. This is also described in Table 3.2, with some additional examples of the expression characters that can be used to describe alleles with variants that impact on gene expression.

The continued use and implementation of the nomenclature has been overseen and reported to the wider audience by the WHO Nomenclature Committee for Factors of the HLA System, who have published a number of official reports describing the process of naming the genes and allelic variants of the *HLA* genes [18, 30, 32–43]. These publications update the wider audience on any new genes officially recognized or any changes to the nomenclature. As discussed, the nomenclature has also changed subtly over its lifetime to reflect the challenges of naming

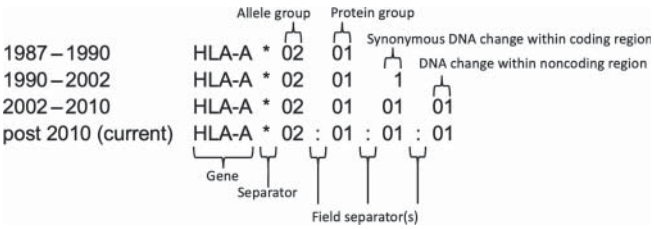

Figure 3.4 The figure details how the initial four-digit name has been expanded overtime and incorporated additional characters to become a four-field name allowing more alleles to be named. The original nomenclature would only allow for up to ninety-nine proteins within each allele group, and while this originally seemed a large number, there are now almost 1,200 HLA-A*02 proteins.

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Table 3.2 Examples of the HLA nomenclature. The table shows examples of how the nomenclature can be used to represent different forms of variation.

Nomenclature	Indicates
HLA	the HLA region and prefix for an HLA gene
HLA-DRB1	a particular HLA locus, *i.e.* DRB1
HLA-DRB1*13	a group of alleles that encode the DR13 antigen or sequence homology to other DRB1*13 alleles
HLA-DRB1*13:01	a specific HLA allele
HLA-DRB1*13:01:02	an allele that differs by a synonymous mutation from DRB1*13:01:01
HLA-DRB1*13:01:01:02	an allele which contains a mutation outside the coding region from DRB1*13:01:01:01
HLA-A*24:09N	a "Null" allele – an allele that is not expressed
HLA-A*30:14L	an allele encoding a protein with significantly reduced or "Low" cell surface expression
HLA-A*24:02:01:02L	an allele encoding a protein with significantly reduced or "Low" cell surface expression, where the mutation is found outside the coding region
HLA-B*44:02:01:02S	an allele encoding a protein which is expressed as a "Secreted" molecule only
HLA-A*32:11Q	an allele which has a mutation that has previously been shown to have a significant effect on cell surface expression, but where this has not been confirmed and its expression remains "Questionable"

Source: Adapted From James Robinson.

increasing numbers of alleles, and Figure 3.5 shows key milestones in how the nomenclature and IPD-IMGT/HLA Database, see section 3.5, has developed over the past 50+ years.

As discussed, the HLA nomenclature was established in 1967, predating many of the more recent attempts to curate sequence variation, and as a result, it differs from other variant reporting format but has shown to be appropriate and highly suited to HLA. In some fields, variants are often reported by simply listing the position and change [44, 45], for example, the *HLA-A*01:02:01:01* allele sequence could be described as

"HLA00001.1:c.[98T>C; 121C>A; 123C>T; 126G>A; 144C>A]",

where HLA00001.1 refers to the coding sequence of the *HLA-A*01:01:01:01* allele. This is a simple description, just five substitutions; however, when considering the number of alleles and the level of variation seen, the number of descriptors required rapidly rises to hundreds of thousands just for a single gene. To highlight this, Table 3.3 shows the extent of variation seen in the *HLA-A*02* group between the initial and recent releases of the IPD-IMGT/HLA Database. For this reason,

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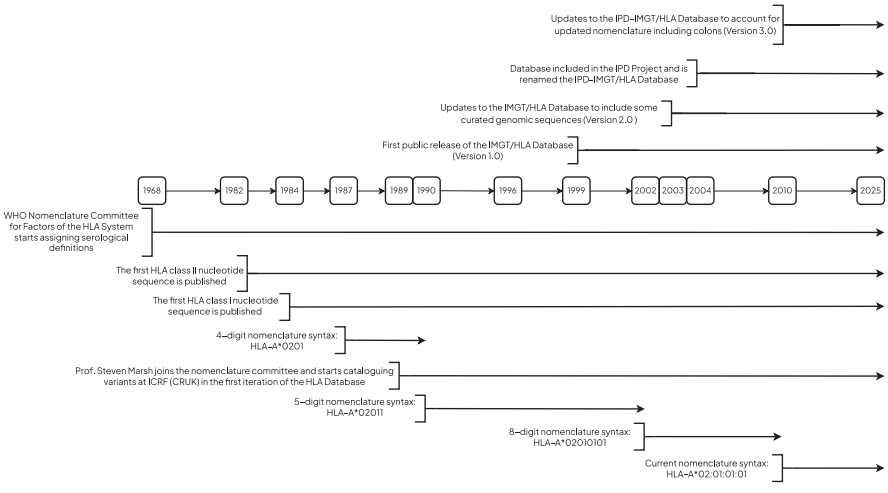

Figure 3.5 A simplified timeline of key events in the implementation of the HLA nomenclature and accompanying HLA Databases.

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Table 3.3 Growth of alleles over twenty-five years. The growth in the number of *HLA* alleles over the last 25 years can be seen by the increased numbers of *HLA-A*02* alleles and proteins since 1998.

	Release 1.0.0	Release 3.57.0
HLA-A alleles	105	8,381
HLA-A*02 alleles	28	1,892
HLA-A*02 proteins	26	1,181
HLA-A*02:01 alleles	2	474
HLA-A*02:01:01 alleles	1	255

descriptive methods of detailing variation like that used by the Human Genome Variation Society or reporting formats like variant call format are less commonly used with HLA. While there are HLA entries in systems that use these methods, these databases are often incomplete and do not accurately reflect true *HLA* variation and many do not contain details of all officially named *HLA* alleles.

3.1.10 Practical Usage of the Nomenclature

HLA nomenclature is now a core component of reporting *HLA* typing results, and the usage of it has developed its own implementations and practices. While the nomenclature can be used to describe a single DNA sequence, the methods used to identify the *HLA* type of a sample can often provide ambiguous results, and these interpretations can lead to challenges in applying and storing the data.

3.1.10.1 Ambiguous *HLA* Types and Typing Strings

The ability of different *HLA* typing methods to determine whether the variation identified was in phase, *i.e.* found within the DNA sequence of the same allele, differs. A solution has been to report all combinations of alleles that were found to occur based on the data generated. Table 3.4 details different reporting methods for the *HLA-A*02:10/HLA-A*02:453* ambiguity. These two alleles share the same sequence over exons 2 and 3, and as a result, depending on the sequencing method performed, any method that didn't include additional regions of the gene would not be able to tell them apart. The data and the typing would be reported as an ambiguous typing string, *HLA-A*02:10/02:453*. These strings of allele names can become very long and complicated. To aid in both readability and suitability for electronic data storage, a number of shorter forms were developed. The methods provide a more compact and alternative method of describing allele strings.

3.1.10.2 G, P, and Other Groups

The official HLA nomenclature has a list of G groups [43]. This designation consists of a three-field allele name followed by an uppercase G. It represents all HLA alleles

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Table 3.4 Alternative ways of encoding an ambiguous HLA typing results. Examples of different descriptors for the same ambiguous sequence of *HLA-A* for exons 2+3.

Encoding	Example String
HLA allele string	A*02:10/A*02:453 or A*02:10/02:453 or A*02:10/453
G Group Nomenclature	A*02:10:01G
MAC designation	HLA-A*02:ZAKU

that have identical nucleotide sequences across the exons encoding the peptide binding domains (exons 2 and 3 for HLA class I, and exon 2 only for HLA class II alleles). For example, as mentioned above, the *HLA-A*02:10:01G* group includes the alleles *HLA-A*02:10 and HLA-A*02:453*.

There are other designations that are also used including the P groups, which is part of the official HLA nomenclature and details alleles with identical expressed proteins in the peptide binding groove, for example, HLA-A*02:10P.

3.1.10.3 NMDP Multiple Allele Designations

A second recoding system is the NMDP (formerly the National Marrow Donor Program) Multiple Allele Code (MAC) Designations (https://hml.nmdp.org/MacUI/). These were developed as the increasing *HLA* diversity led to ever longer typing strings and was deployed as a shorthand for reporting these types. The MAC Designations are colloquially referred to as NMDP codes or MAC codes and comprise the gene name followed by a first field numerical code and a series of two to five letters (the MAC code), which summarizes all the combinations of *HLA* alleles observed within the string. This system is designed for encoding combinations of alleles over the exon 2+3 sequence of HLA class I and exon 2 of HLA class II genes. It is primarily aimed at two-field allele designations and is not designed for encoding ambiguity at just the third or fourth field. For example, the MAC Designation *HLA-A*02:ZAKU* is comprised of the gene and the first field designation followed but the MAC code ZAKU, which equates to "10/453". While there are MAC Designations representing G groups, these are updated with each release of the IPD-IMGT/HLA Database as the G group expands in size. This means that there are potentially multiple MAC Designations representing the same shared sequence depending on when they were defined.

3.1.11 Reporting Different Resolution Levels

The HLA nomenclature is designed to provide a unique designation for each allele. The nomenclature also allows the representation of groups of alleles based on common fields. This is most often seen when reporting alleles to a certain field level. For example, *HLA-A*02* could be described as first field resolution and would refer to all alleles starting *HLA-A*02*. A second field resolution would be *HLA-A*02:01*, referring to all alleles starting *HLA-A*02:01*.

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Previously, these resolutions were often referred to as two- or four-digit resolution, but this terminology should be avoided as some alleles have three- or four-digit designations at the first or second field. An important distinction, and a sometimes-misinterpreted view, is that a two-field designation refers to a partial sequence, a three-field designation refers to a full-length coding sequence, and a four-field designation refers to a genomic sequence. This is not correct. While the resolution defines where variation lies, it is does not define the length of sequence represented by the allele name. As of release 3.57.0 (July 2024), there are 8,190 alleles with a two-field name that have a full genomic sequence, and 3,714 alleles with a three-field name that do not have a full coding sequence.

In addition, describing sequences as matching or being identical or accurate to a set field resolution can be misleading. Reporting two individuals as having identical sequences to the second field merely indicates that they share an assigned allele name to the sequences produced, and not sequence identity. For example, *HLA-A*02:01* refers to 473 distinct nucleotide sequences of different lengths that all encode the same protein sequence. Therefore, only using the abridged nomenclature does not differentiate between the many variants identified. Matching can be performed at different levels if you acknowledge the caveats above.

3.1.12 Analyzing Data Based on Geographical or Ancestral Information

The reporting of HLA typing and potential review of any ambiguous strings can also be influenced by the frequencies of known HLA alleles in any given demographic. It has long been established that the frequency of different HLA alleles shows widespread variation across human populations.

Importantly, allelic and haplotypic frequencies of the HLA genes can vary dramatically across human populations. This is likely due to the central role of HLA in immune responses, which means the HLA system may be influenced by both demographic and selective mechanisms, in particular balancing selection.

There are a number of resources dedicated to providing HLA frequency data at various levels, as well as resource showing the most commonly seen alleles in different demographics [46–48].

The Common & Well-Defined (CWD) and later Common, Intermediate, and Well-Defined (CIWD) allele lists [49, 50] provide data on the frequencies of different alleles in seven geographic/ancestral/ethnic groups. These lists and the associated websites can help review data based on demographic data as well as typing data. The original CWD lists focused primarily on US populations, but subsequent work has expanded these to cover more populations spread across the globe [48, 51].

3.2 IPD-IMGT/HLA Database

Access to all official sequences named by the WHO Nomenclature Committee for Factors of the HLA System is now primarily through the IPD-IMGT/HLA Database. Initially, the nomenclature reports and associated sequences were published in a number of journals or distributed via floppy disks. With the advent of the internet, a

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new method of data dissemination was available. Initially, sequences were available from the website of the Tissue Antigen Laboratory of the Imperial Cancer Research Fund, UK (now known as Cancer Research UK), and then through the ongoing IPD-IMGT/HLA Database. The only official repository of all known *HLA* sequences is the IPD-IMGT/HLA Database [19, 20, 52], which, as of July 2024, contained 39,627 HLA alleles and a further 996 alleles from HLA-related genes.

The IPD-IMGT/HLA Database maintains a high-quality reference system of manually curated and expertly reviewed sequences. The curation of the sequences has been paramount to the success of the model. While the construction of a database of HLA sequences could be performed by simply querying the existing databanks included in the International Nucleotide Sequence Database Collaboration (INSDC) [53, 54], this would not provide the same curated resource. Searching for the term "HLA" provides sequences for 6,339 genome assemblies and a further 198,111 individual sequences covering 111,198 protein sequence (data retrieved from https://www.ebi.ac.uk/ebisearch/about August 2024). However, while this data is curated to ensure minimum standards have been met for inclusion in the INSDC repositories, it is not curated with a specific focus on HLA. Within the INSDC entries, keywords are assigned by the submitters, and these can vary. *HLA* allele names are not mandatory, and there can be multiple copies of the same sequences deposited. Different sequences can share the same keywords and allele assignments, and identical sequences can have differing keywords and different allele assignments. While these generalist repositories provide a wealth of information for many areas of study, for those working on HLA, the accurate curation, maintenance, and publication of the sequences as in the IPD-IMGT/HLA Database are fundamental.

The IPD-IMGT/HLA Database also provides users with a number of tools for searching and downloading the data on both the nomenclature and sequences of known *HLA* alleles, as well as additional tools for *HLA* analysis.

3.3 Limits to HLA Variation

The number of new submissions received by the IPD-IMGT/HLA Database continues to grow, and the number of alleles named each year remains high (Figure 3.3). As a result, an often-asked question is "How many HLA alleles are there, and have we seen them all?". Studies by Robinson *et al.* [21] and Klitz *et al.* [55, 56] used different models to predict the maximum number of alleles over just the antigen recognition domain. These theoretical predictions suggest that there could be as many as 2.7×10^6 *HLA-A*, 3.3×10^6 *HLA-B,* and 3.2×10^6 *HLA-C* alleles.

Using the same methodology for HLA class II, the number of HLA class II alleles is likely to lie between 0.7 and 1.7 million alleles for the HLA class II β-chain genes, while the more conserved HLA class II α-chain genes show theoretical numbers of between 5,000 and 80,000 variants.

These numbers focus on the antigen recognition domain, and as more samples are sequenced for the full gene, more polymorphism is revealed, meaning the true scale is likely to be higher than the numbers previously estimated.

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3.4 Summary

The HLA gene system is a highly complex and polymorphic system fundamental to our immune response and of high clinical relevance. The study of this region has been supported by the development of a detailed nomenclature system designed not only to identify variants but also provide some functional information. The dissemination of the nomenclature and resources for studying the sequences are provided through the IPD-IMGT/HLA Database.

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4

The HNA System

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4.1 Neutrophils and Associated Functions

4.1.1 The Neutrophil

Granulocytes are the major component of White Blood Cells (WBC) and form an essential role in the immune system to control infection from pathogens. These cells possess a multi-lobed nucleus and a distinctive granular cytoplasm due to the possession of numerous granules that contain immune mediators involved in the clearance of pathogens. Granulocytes include several cell types, which are the Neutrophils, Basophils, Eosinophils, and Mast cells, each with specialized roles in immune defense. Neutrophils are the major constituent of Granulocytes, accounting for over 90% with an adult reference range of $1.8–7.5 \times 10^9$/l. Mild neutropenia has been defined as $1–1.5 \times 10^9$/l, and severe neutropenia $<0.5\ 10^9$/l. Less than $0.2–0.5 \times 10^9$/l is associated with increased risk of complications from life-threatening infections, which may be associated with both sepsis and pneumonia [1, 2]. Reference ranges have also been established for neonates with Neutropenia defined as $<1.0\ 10^9$/l for 14 days up to 12 months of age [3]. Reference ranges for less than 14 days of age are difficult to establish due to high variation in cell counts [3]. It must also be noted that some adult reference ranges may vary depending on the ethnic origin of the population, specifically, some Middle Eastern and Black African populations have a lower adult reference range [3, 4].

Neutrophils are produced in the bone marrow from myeloid progenitor cells, then undergo maturation through several stages: myeloblast, promyelocyte, myelocyte, metamyelocyte, band cell, and finally develop into a polymorphonuclear mature cell. Mature Neutrophils circulate in the blood system with an estímated half-life of nineteen hours, although one study suggested 5.4 days [5]. Neutrophil's main function is to protect against infectious agents as part of the innate immune system. Neutrophil activity against microorganisms includes phagocytosis, degranulation and production of neutrophil extracellular traps (NETs) [6].

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Previously described as only a terminally differentiated cell with limited but essential functions. Neutrophil heterogeneity in phenotype and various functions that bridge both innate and adaptive immunity have been reported [7].

4.1.2 Causes of Neutropenia

The most common cause of neutropenia is an active infection, or drug induced neutropenia (DIN) which includes anti-leukemic drugs and drugs, such as carbamazepine (anti-epileptic), sulfasalazine (anti-thyroid), and clozapine (neuroleptic). DIN has been reported to have a combined incidence in Europe of between 1.6 and 9.2 cases per million population [4, 8]. Reduction in neutrophils can also be caused by both inherited disorders and diseases that adversely affect or interfere with neutrophil function and production. In adults' neutropenia may be secondary to bone marrow diseases including leukaemia, aplastic anaemia, and lymphoma. Secondary neutropenia may also be caused by nutritional deficiencies, such as Folate and Vitamin B12 [9]. Congenital Neutropenia includes a variety of rare syndromes with inheritance of mutations with autosomal recessive, autosomal dominant and X-linked modes of inheritance. Some examples of severe congenital neutropenia are included in the umbrella term Kostmann Syndrome associated with mutations in *ELANE, GFI1, HAX1, G6PC3, VPS45 and WASP* genes [4, 8, 9].

Immune destruction of neutrophils is associated with antibodies that specifically target cell surface molecules that possess immunogenic epitopes. In rare occasions the lack of a glycoprotein receptor can also lead to individuals producing antibodies when exposed to the antigen they lack. Both these examples are relevant to the production of Neutrophil specific antibodies [10]. Allogeneic production of antibodies may be IgM or IgG epitope specific and are associated with Alloimmune neutropenia, whilst Autoimmune neutropenia may be associated with either IgG and IgM self-reactive antibodies which may be glycoprotein specific or epitope specific. Where individuals lack an antigen they may produce an Iso-antibody which will be glycoprotein specific rather than epitope specific [10].

4.2 Human Neutrophil Antigens (HNA)

Neutrophil functions through the ability of specific glycoprotein complexes that have specificity for ligands, which enable the neutrophil to fulfil various functions, such as binding to IgG Fc receptors to detect Immunoglobulin (Ig) labelled pathogens. The interaction of the CD16b receptors with Ig promotes neutrophil activation [11].

There are nucleotide changes that produce amino acid exchanges within some of the neutrophil associated glycoproteins. These amino acid changes are due to specific polymorphisms which are the basis of the HNA system [10, 12]. Whilst most of the HNAs do not affect the function of the glycoprotein, they can act as an antigenic epitope that can promote an antibody response [10]. Some of the glycoproteins where HNA epitopes are located may be granulocyte specific whilst others are also expressed by several cell types [10, 12].

HNA nomenclature is governed by the International Society of Blood Transfusion Granulocyte Immunology Working Party (ISBT GIWP) [13, 14]. Currently, there are

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five antigen systems, HNA-1 through to -5, consisting of fourteen defined alleles [13, 14].

4.2.1 HNA-1

HNA-1 is subdivided into four distinct epitopes: HNA-1a, -b, -c, and -d, located on the CD16b glycoprotein. The CD16b receptor is also referred to as FcyIIIb, which is a low-affinity IgG Fc receptor; it is the only Fc receptor attached to the cell membrane by a glycosylphosphatidylinositol (GPI) linker [11, 12]. Key functions of FcγIIIb (CD16b) include induction of actin polymerisation, and mediating calcium rise through Transient Receptor Potential Cation Channel Subfamily M Member 2 (TRPM2) and NETs formation [11, 12]. FcγIIIb (CD16b) acts with FcγIIIa (CD16a) to induce degranulation, phagocytosis and oxidative burst to aid the eradication of pathogens. CD16b expression is limited to neutrophils, whilst CD16a is expressed by macrophages and NK cells. CD16a and CD16b share a high degree of sequence homology, however, CD16a has been reported to possess a 10-fold greater affinity for IgG when compared to CD16b [12].

CD16b is encoded by the gene *FCGR3B,* which is located on chromosome 1q23-24. The gene has five exons that encode a 233 amino acid peptide that includes an 18 amino acid signal peptide sequence. All HNA-1 associated polymorphism is limited to exon three, which forms part of the membrane distal domain that binds to the Fc region of IgG molecules [12].

In accordance with the ISBT GIWP [13, 14] allele names are integrated into the nomenclature using the gene name hence, HNA-1a is defined as *FCGR3B*01* then additional alleles are numbered sequentially. When considering inheritance of HNA-1 alleles, it must be noted that there may be gene copy number variation; an individual may have no alleles (null phenotype) or up to four alleles [12]. The variation in copy number has been proposed to be due to gene duplication, recombination and unequal cross-over during meiosis [12].

The HNA-1 antigens are formed by five key nucleotide substitutions that encode the relevant amino acid exchanges at positions 36, 65, 78, 82, and 106, numbering refers to the *FCGR3B* transcript variant two (NM_000570.4) [12–14]. Currently, to date there are five HNA-1 alleles, and an additional allele that indicates null expression (Table 4.1). HNA-1a is encoded by the *FCGR3B*01* allele this was the first described HNA and was formerly named NA1, antibodies specific for HNA-1a have been indicated in clinical conditions, such as Neonatal alloimmune neutropenia (NAIN), Autoimmune neutropenia (AIN), Drug-induced neutropenia (DIN) and Transfusion related acute lung injury (TRALI). The clinical associated conditions will be discussed later in the Chapter. HNA-1a frequency has been reported to be as high as 90% in Asian populations, compared to around 60% in Caucasoid and Black African populations (Table 4.2). The HNA-1a antigen is also encoded by the *FCGR3B*04* allele, the only difference between this allele and *FCGR3B*01* is a single nucleotide change at position 316G>A leading to an amino acid exchange at position 106 from Valine to Isoleucine (Table 4.1). Antibody reactivity to the *FCGR3B*04* allele has been reported to be the same as the *FCGR3B*01* allele.

HNA-1b previously referred to as NA2 is encoded by both *FCGR3B*02* and *FCGR3B*03* alleles, as shown in Table 4.1. Antibodies directed against the HNA-1b

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Table 4.1 Nucleotide and amino acid positions that form the different HNA-1 epitopes.

Allele/Antigen	Nucleotide and Amino acid positions				
*FCGR3B*01* HNA-1a	*108 G* Arginine 36	*194 A* Asparagine 65	*233 C* Alanine 78	*244 G* Aspartic acid 82	*316 G* Valine 106
*FCGR3B*02* HNA-1b & HNA-1d	*108 C* Serine 36	*194 G* Serine 65	*233 C* Alanine 78	*244 A* Asparagine 82	*316 A* Isoleucine 106
*FCGR3B*03* HNA-1b & HNA-1c	*108 C* Serine 36	*194 G* Serine 65	*233 C* Aspartic acid 82	*244 A* Asparagine 82	*316 A* Isoleucine 106
*FCGR3B*04* HNA-1a	*108 G* Arginine 36	*194 A* Asparagine 65	*233 C* Alanine 78	*244 G* Aspartic acid 82	*316 G* Isoleucine 106
*FCGR3B*05* HNA-1b var[a)]	*108 G* Serine 36	*194 A* Serine 65	*233 C* Alanine 78	*244 G* Aspartic acid 82	*316 G* Isoleucine 106
FCGR3B null* CD16b-null	Individuals because of a genetic mutation in the *FCGR3B gene,* lack CD16b expression and are referred to as CD16b null				

Note one allele can encode for more than one antigen.

a) HNA-1b antibody reactivity to this allele has been reported to be weaker compared to other HNA-1b alleles/antigens.

antigen are associated with the same clinical conditions as HNA-1a but to a lower frequency than HNA-1a. *FCGR3B*02* differs from *FCGR3B*01* in all but one of the five key amino acid positions where there are specifically several amino acid exchanges Arg36>Ser, Asn65>Ser, Ala78, Asp82>Asn, and Val106>Ile (Table 4.1). In addition, the *FCGR3B*02* allele carries the HNA-1d epitope that is formed by the possession of both Alanine at position 78 and Asparagine at position 82, color coded amber to aid interpretation in Table 4.1. Antibodies specific for HNA-1d antigen were first described in a NAIN case [10, 12] where the implicated antibodies were demonstrated to have specificity for *FCGR3B*02* positive blood donors and negative for *FCGR3B*03* positive blood donors. The HNA-1c epitope is located on the *FCGR3B*03,* where this allele differs from the *FCGR3B*02* allele by only a single amino acid exchange, Ala78Asp (Colour coded blue see Table 4.1). There is a lower

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Table 4.2 HNA phenotype frequencies in different populations.

Population	Chinese	British	German	Danish	African
Phenotype					
HNA-1a/1a	43–46 (A)	8	15	13	19
HNA-1b/1b	9 (B)	43	36	42	16
HNA-1c/1c	0	0	0	0	12 (C)
HNA-1a/1b	44–48 (A)	46	48	39	25
HNA-1a/1c	0	0	0	2	15
HNA-1b/1c	0	1	0	0	10
HNA-1a/1b/1c	0–1	2	1	4	4
CD16 null	0.5	0–1	0–1	0–1	1–7 (C)
HNA-3a/3a	51–53	56	56	67	95 (D)
HNA-3a/3b	39–41	41	38	29	5
HNA-3b/3b	6–9	3	7	4	0
HNA-4a/4a	99 (E)	79	83	78	81
HNA-4a/4b	1	19	15	21	17
HNA-4b/4b	0	2	2	1	2
HNA-5a/5a	72–73	54	56	52	22
HNA-5a/5b	24–27	39	35	39	56
HNA-5b/5b	1–2	7	9	8	22 (F)
CD177 null	1–11	3–13	3–13	3–13	2

Adapted from Bux (2008), Nielsen (2012), and Xia (2011) [15–17].
A = Chinese population have a higher HNA1a frequency compared to Caucasoid & African populations – conversely HNA-1b is at a lower frequency in the Chinese population (B) compared to Caucasoid populations.
C = African populations have greater frequency of HNA-1c and of CD16 null – upto 7% thus more likely to detect CD16 Isoantibodies in this population.
D = HNA-3a is at highest frequency in African population but lack HNA-3b3b – thus extremely unlikely to have patient from African population that could produce an HNA-3b antibody.
E = HNA-4b is almost absent in Chinese population and thus not likely to produce HNA-4b antibody when compared to other populations.
F = HNA-5bb is much more frequent in African population therefore higher chance of patient with HNA-5a antibody compared to other populations.

frequency of cases with antibodies directed to HNA-1d and HNA-1c, and these are mostly associated with NAIN [10, 12]. HNA-1c is a low-frequency antigen in most populations apart from in Black African populations (See Table 4.2). The HNA-1b variant allele *FCGR3B*05* has been reported to have lower antibody reactivity when compared to other HNA-1b alleles [12–14].

The *FCGR3B*null* allele relates to the CD16b null phenotype, which may result in individuals producing an anti-CD16b isoantibody and is a low frequency allele with higher frequency seen in Black African populations, while almost absent in other populations (See Table 4.2), [12].

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4.2.2 HNA-2

HNA-2 is a simple antigen system with expression of the antigen or a null phenotype, leading to the potential to produce isoantibodies against the antigen that the individual lacks, in this case, CD177. The CD177 acts as a receptor for the platelet endothelial cell adhesion molecule 1 (PECAM-1), which may be linked to neutrophil transmigration [18]. The gene that encodes the CD177 glycoprotein is located on chromosome 19q13.31 and has nine exons that encode a 437 amino acid protein, which includes a 21 amino acid signal peptide sequence [10, 12].

CD177 was previously described as NB1, with associated antibodies detected in a NAIN case [10, 12]. Detection of HNA-2 (CD177) specific antibodies exhibits a bi-modal pattern of expression, which reflects the distribution of expression of CD177 that can differ between specific sub-populations of neutrophils. The differential expression levels have been described to be consistent per individual. Post-infection or administration of G-CSF, CD177 expression has been shown to increase as neutrophil development and activation are stimulated.

There have been several reports of various genetic mutations that may result in a null phenotype, frequency of the resultant CD177 null phenotype is low and ranges from three to 13% in Caucasoid populations (see Table 4.2), around 2% in African and up to 11% in Southeast Asian populations [15–17].

4.2.3 HNA-3

HNA-3 is an antigen that is expressed on the Choline transporter-like protein two (CTL2) molecule, there are two epitopes, namely HNA-3a, which is formed by the presence of Arginine at position 152, and HNA-3b is formed by the possession of Glutamine at position 152, located in the first extracellular loop of CTL2 [10, 13, 14]. CTL2 is encoded by the *SLC44A2* gene located on chromosome 19p13.1. The gene has 22 exons, which encode 706 amino acids, and there are two known transcription variants, TV1 (NM_020428.3) and TV2 (NM_001145056.1). TV1 is slightly longer and has been reported to support Choline transport through the cell membrane. TV2 expression has been described to include Neutrophils, Lymphocytes, platelets, and liver tissue, while both TV1 and TV2 are expressed by lung tissue, and endothelium in various tissues, such as liver, colon and the inner ear [10, 12–14].

HNA-3a has been assigned the allele *SLC44A2*01* (G at position 455) and HNA-3b *SLC44A2*02* (A at position 455), which results in the Arg152Gln exchange [13, 14]. In addition to the single nucleotide polymorphism (SNP) responsible for the *SLC44A2*01/02* alleles there is a third allele which has been assigned *SLC44A2*03* this is a result of a C>T substitution at nucleotide 451 resulting in an amino acid exchange of Leu151Phe, possession of this epitope has been associated with reduced HNA-3a antibody reactivity [12–14]. Antigen frequency can be seen in Table 4.2, HNA-3a is the most common antigen while 3b has considerably lower population frequency, a minority (5%) of Caucasoids possess the HNA-3b3b phenotype and are at risk of developing anti-HNA-3a antibodies [15–17].

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4.2.4 HNA-4

HNA-4 is an example of a bi-allelic antigen system with two epitopes assigned HNA-4a and HNA-4b. The HNA-4a/b antigen is located on the integrin alpha M chain (ITGAM), also referred to as CD11b [12–14]. The CD11b glycoprotein chain associates with CD18 (ITGB3) beta Integrin chain to form the heterodimer known as the macrophage receptor (Mac-1) or integrin αMB_2. Mac-1 is highly expressed on Neutrophils, but not limited to just this cell type but also expressed by mast cells, cytotoxic T-cells, fibrocytes, and Natural Killer cells. CD11b/CD18 (Mac-1) resides in a low-affinity state on the cell surface membrane until cell activation, upon which the integrin transitions into a higheraffinity state to aid neutrophil transmigration, phagocytosis, and oxidative burst. MAC-1 acts as a complement receptor and is also referred to as CR3 [10, 12].

The gene that encodes the CD11b alpha chain is *ITGAM*, located on Chromosome 16p11.2. The transcribed protein has a 1,153 amino acid sequence which includes a 16 amino acid signal peptide. The signal peptide is cleaved to produce the mature alpha chain peptide (NM_001145808.1, TV1). HNA-4a has been assigned *ITGAM*01* and HNA-4b assigned *ITGAM*02* these are associated with the SNP *ITGAM*230G>A* (rs1143679) which leads to an Arg61His amino acid exchange [12–14]. Phenotype frequencies can be seen in Table 4.2, like HNA-3a, HNA-4a is the higher frequency phenotype either in Homozygous HNA-4a/4a or heterozygous state HNA-4a/4b, with HNA-4b/4b in lower frequencies in most populations and almost absent in the Chinese population [15–17].

4.2.5 HNA-5

HNA-5 is similar to HNA-4, where the antigen is located on a glycoprotein chain, namely CD11a, which forms a heterodimer with CD18 to form, in this case, the Lymphocyte Activating Factor-1 (LAF-1) molecule. CD11a is also referred to as the Integrin alpha L chain (ITGAL) encoded by the *ITGAL* gene. The *ITGAL* gene is located on chromosome 16 at position 16p11.2, has thirty-one exons, which translates to a 1,170 amino acid precursor peptide, which is inclusive of a 25 amino acid signal sequence that is cleaved to produce the mature peptide [10, 12–14]. LAF-1 acts as a receptor for intracelular adhesion molecules (ICAM), which are involved in endothelial transmigration, and are also expressed on all Leukocytes.

HNA-5a refers to the allele *ITGAL*01 where* the 2372G nucleotide results in amino acid Arginine at position 766, the anithetical antigen is associated with the SNP *ITGAL*02* (2372G>C, Arg766>Thr) [13, 14]. Currently, antibodies have been detected only to HNA-5a with no reported cases of antibody response to the antithetical antigen and thus remains absent from the nomenclature [13, 14]. The majority of HNA-5a antibodies have been limited to association with NAIN [10, 12].

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4.3 Clinical Conditions Associated with HNA Specific Antibodies

4.3.1 Neonatal Alloimmune Neutropenia (NAIN)

Neutropenia associated with HNA-specific antibodies dates to the early 1950s, and a decade later a key publication by Lezari *et al* in 1960 [19] described a case of Neonatal neutropenia due to maternal immunization, which was one of the first of many publications describing NAIN [10, 12, 20, 21]. NAIN is caused by maternal HNA-specific IgG antibodies crossing the placenta via the fetal Fc receptor (FcRn) resulting in destruction of neonatal neutrophils. Incidence has been reported to be <1/1,000 live births, and symptoms range from none to skin infections, omphalitis, and the more severe complications of neutropenia resulting in pneumonia and sepsis [20, 21]. Most HNA antibody specificities that have been associated with NAIN are the more common epitope allo-specific antibodies that are directed against HNA-1a, HNA-1b, and to a lesser extent, iso-antibodies to both HNA-2 and CD16b [10, 12, 20, 21]. There is a lower frequency of reported cases with detection of IgG allo-antibodies specific for HNA-1c, HNA-1d, HNA-4a, HNA-4b and HNA-5a [10, 12].

Treatment of affected newborns usually involves antibiotics to combat any infections, this may be supplemented with Granulocyte-Colony Stimulating Factor (G-CSF) to increase neutrophil counts, and also IVIG in more severe cases [4]. In the majority of cases, the neutropenia and associated symptoms will recover in three to four months [4].

4.3.2 Autoimmune Neutropenia (AIN)

Autoimmune neutropenia is associated with the development of antibodies that are directed to autologous/self HNA specific epitopes or Glycoprotein complexes, such as CD16b [10, 22]. These antibodies may be IgG or IgM with a defined specificity or Neutrophil (Granulocyte) specific that fail to be defined any further. Antibody detection aids differential diagnosis by confirming an autoimmune pathology versus an alternative cause of Neutropenia, such as congenital disorders or diseases/treatment that can reduce Neutrophil production [4, 22, 23]. AIN is subdivided into primary neutropenia, usually associated with Neutrophil antibodies, and Secondary AIN, which refers to Neutropenia as a development due to a preexisting condition, such as rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE), to name but a few [4, 22, 23].

ANI occurs more frequently in pediatric patients less than 3–4 years old and is usually associated with HNA-1a, HNA-1b, and anti-CD16b antibodies. Incidence of ANI has been reported to be 1 in 100,000 of children less than 10 years old, which may last months but usually resolves within 24–36 months [4, 24]. Treatment will depend on clinical presentation if active infection is present, antibiotics are indicated, which may be supplemented with G-CSF, steroids, and rarely intravenous immunoglobulin (IvIG) [4, 23, 24].

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4.3.3 Complications of Transplantation

Neutrophil antibodies have been associated with post stem-cell transplant neutropenia, specifically presenting as delayed engraftment, failed engraftment and or rejection [25–27].

The presence of HNA incompatibility is not a veto to stem cell transplantation, however prior knowledge may aid more informed clinical management.

HNA-3a/3b antibodies have been reported to be associated with humoral rejection in kidney transplantation, in several publications [28–30] have supported this incidence, which was prompted by investigation of positive lymphocyte crossmatches in the absence of HLA donor-specific antibodies. The positive crossmatch is explained by the expression of HNA-3a/3b on lymphocytes and endothelial tissue. Confirmation by detection of HNA-3a or HNA-3b specific antibodies and HNA-3 incompatibility between the donor and recipient [28–30].

4.3.4 Complications of Transfusion

HNA antibodies are associated with several complications of transfusion that include febrile non-hemolytic Transfusion Reaction (FNHTR), TRALI, and transfusion-related alloimmune neutropenia (TRAIN). FNHTR and TRALI are covered in detail in the Transfusion Medicine section of this Textbook (Chapter 14). TRAIN is a rare complication of transfusion where HNA antibodies are transfused in blood products, such as fresh-frozen plasma and pooled platelets, and the antibodies react with the HNA cognate antigen possessed by the recipient, which subsequently results in neutropenia [31].

4.3.5 Drug-Induced Neutropenia (DIN)

DIN is caused by the production of drug-dependent antibodies (DDAbs) that are directed against neutrophil-specific glycoproteins. There are several examples of drugs that have been associated with an immune-mediated neutropenia, which is also referred to as drug-induced immune neutropenia (DIIN) or idiosyncratic drug-induced neutropenia (IDIN). These include Quinine, Carbimazole, Penicillins, Cephalosporine, Lactamine, and Flecanide [4, 8, 23]. In a review by Brian Curtis [8], incidence of non-chemotherapy IDIN ranges from 1.6 to 15.4 cases per million population [8].

There are two proposed immune mechanisms to explain DIN/DIIN which are a Hapten-based hypothesis and a Danger signal hypothesis [8]. The hapten hypothesis suggests that the drug, which should be of low-molecular weight, binds non-covalently to a protein, in the case of DIN a neutrophil-specific glycoprotein.

The Danger hypothesis of DIN proposes that Antigen Presenting cells (APCs) present the hapten-bound glycoprotein complex to T helper (Th) cells, as the initial stage or signal one. Signal two, which is required for APC activation, involves co-stimulation via B7 and CD28 with the Th cells, which results in production of the antibodies that recognize the neutrophil-specific antigen in combination with the drug or drug metabolite [8].

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In confirmed cases where there is a demonstrable immune cause of DIN implicated in the presence of a specific drug, the obvious treatment is cessation of the implicated drug unless there are no alternative therapeutic agents [23].

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5

Natural Killer Cell Receptors

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5.1 Introduction

The immune system can be divided into two subsystems: adaptive immune system and innate immune system. The adaptive immune response is highly specific for a pathogen, and the response improves with each successive encounter with the same pathogen. The innate immune system represents the first line of defense against tumor cells and bacterial and viral infections. The immune response generated by this system is rapid, has no memory, and is nonspecific [1]. The cells primarily involved in the adaptive immune response are T and B cells. The cells of the innate immune system include natural killer (NK) cells, phagocytes, mast cells, and dendritic cells. The major molecules that play a role in the innate system are those of the complement system and cytokines. For example, the molecule mannan-binding lectin of the complement system that recognizes molecules containing mannose on the surface of bacteria helps activate the complement cascade. NK cells, through receptors on the cell surface, recognize and induce the lysis of tumor cells or cells infected with viruses that have altered major histocompatibility (MHC) molecules or have downregulated MHC expression to evade T cell-mediated responses. NK cells also produce cytokines [interferon (IFN)-γ, tumor necrosis factor (TNF-α)] and chemokines (MIP-1, RANTES) that are involved in the destruction of intracellular pathogens. The focus of this review is NK cells and the receptors on these cells that enable killing of tumor and pathogen targets.

5.2 NK Cells

NK cells are bone marrow-derived lymphocytes that are thought to originate from the same lineage as T cells [2]. NK cells are large granular lymphocytes derived

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from $CD34^+$ hematopoietic progenitor cells that mature in the bone marrow. NK cells comprise approximately 10–15% of all circulating blood lymphocytes and can be found in the spleen, lymph nodes, liver, lung, intestine, and placenta. They are characterized by the expression of CD16 and CD56 and the lack of CD3 [3]. The cell surface density of CD56 expression subdivides NK cells into two distinct groups. The majority (90%) of NK cells are $CD56^{dim}$ and express high levels of CD16, while the remaining NK cells are $CD56^{bright}$ and express low levels or no CD16 [4]. The two groups also differ in function and NK receptor repertoires. The $CD56^{dim}$ cells mediate antibody-dependent cellular cytotoxicity (ADCC) and express both killer immunoglobulin-like receptor (KIR) and C-type lectin NK receptors. The $CD56^{bright}$ cells produce cytokines and express high levels of C-type lectin receptors, with only a small number of cells expressing KIR [4, 5]. The main function of NK cells is to kill cells infected with pathogens and tumor cells. NK cells recognize and lyse target cells either by ADCC or natural cytotoxicity [6]. CD16 (FcγRIII) is a receptor that recognizes the Fc portion of IgG antibodies bound to the surface of infected cells and mediates lysis of target by ADCC. The mechanism of killing is identical to that of cytotoxic T cells, and perforins and granzymes are released onto the infected cells surface, where perforin acts in a manner similar to that of complement, creating pores in the cell membrane and allowing granzymes to enter inducing apoptosis. NK cells can also induce apoptosis via surface Fas ligands and TNF receptors. The expression of Fas ligand is increased upon activation of NK cells. These ligands then bind to Fas on target cells triggering killing. NK cells secrete cytokines, mainly IFN-γ, which stimulate T_H1 cells. Natural cytotoxicity does not require antibody and is governed by a balance of inhibitory and activating signals received via receptors on the NK cell interacting with ligands on target cells. NK cells have been shown to discriminate between different target cells, but unlike T and B cells, NK cells do not rearrange genes encoding receptors for antigen recognition. NK receptors are encoded by two gene complexes on different chromosomes in man: the natural killer complex (NKC) on chromosome 12 and the leukocyte receptor complex (LRC) on chromosome 19. The receptors of the NKC and LRC serve similar functions; however, the receptors have distinct structures. Both complexes encode receptors with inhibitory function and counterparts with activating function and are thought to regulate NK cell function through a balance of inhibiting and activating signals. NK receptors bind to MHC class I molecules to mediate their function (reviewed in [7]).

5.3 "Missing Self" Hypothesis and Hybrid Resistance

The conventional laws of transplantation are based on T cell recognition of histocompatibility antigens. If two inbred parental mouse strains differ in their MHC genotypes, their F1 hybrids will express the MHC antigens of both parents. F1 hybrids should therefore accept grafts from either parent as the T cells in the F1 mouse are tolerant of the MHC antigens encoded by both parental MHC haplotypes while both parents should reject grafts from the F1 hybrid. These laws generally apply,

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but exceptions were discovered for hematopoietic grafts. Rejection of parental bone marrow grafts by (A × B) F1 hosts was first described in mice by Cudkowicz and Stimpfling [8]. This phenomenon was called hybrid resistance. The cells responsible for hybrid resistance were discovered to be of marrow origin, and a number of studies in the early 1970s confirmed these to be NK cells (reviewed in [9]). The importance of class I MHC expression in preventing NK-mediated lysis was demonstrated in a series of experiments using transplantable lymphomas. The "missing self" hypothesis first proposed by Karre and Ljunggren states that an NK cell will lyse a target cell that lacks or has reduced expression of "self" MHC class I molecules. This hypothesis was based on the ability of NK cells to kill mouse tumor cells that lacked expression of MHC class I molecules. In the F_1-hybrid anti-parental resistance model, an H-$2^{b/b}$ homozygous bone marrow transplant is rejected by NK cells in an H-$2^{a/b}$ host. The graft does not express the H-2^{a} class I alleles that are present in the recipient, and this lack of expression is sufficient to cause rejection by NK cells [10].

5.4 NK Cell Receptors

NK cells bind to MHC class I molecules via receptors on the cell surface to mediate their cytotoxic function. Two families of receptors have been described: the C-type lectin-like family and the immunoglobulin superfamily (Ig-SF), which include the killer cell immunoglobulin-like receptors (KIRs) and the natural cytotoxicity receptors (NCRs) [6]. The receptors belonging to the C-type lectin family include the CD94/NKG2 heterodimers, NKG2D in humans and mice, and Ly49 in the mouse. The C-type lectin receptors include both inhibitory and activating receptors that interact with classical HLA-E class on potential targets.

KIR are encoded in the LRC on chromosome 19. Similar to the C-type lectin receptors, the KIR family of receptors includes both inhibitory and activating receptors that interact with MHC class I on potential targets. However, unlike the C-type lectin receptors, the KIR receptors are specific for products of a single HLA-A, -B, and -C allotype, and, therefore, enable NK cells to recognize decreased expression of a particular class I MHC locus [11, 12].

The NCRs—NKp46, NKp44, and NKp30—also belong to the immunoglobulin gene superfamily but are not encoded in the LRC. These receptors are activating receptors that interact with non-MHC molecules expressed on all normal cells for the lysis of virus-infected cells and tumor targets [13], and, more recently, some HLA-DP molecules interact with NKp44 receptors [14]. In individuals infected with human cytomegalovirus (CMV), expression of HLA-DP on effector $CD8^+$ T cells is upregulated and NKp44+ NK cells are activated by HLA-DP+ $CD8^+$ T cell populations in an HLA-DP haplotype-dependent process [15].

An individual NK cell clone does not express all NK receptors, but NK clones may express receptors from more than one receptor family, resulting in NK clones with diverse patterns of receptor expression. The variegated pattern of receptor expression allows different NK cells to discriminate among cells with different patterns of MHC expression [15, 16].

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5.5 Killer Immunoglobulin-Like Receptors (KIRs)

5.5.1 KIR Protein Structure

KIR are type I transmembrane glycoproteins that contain a leader sequence, either two or three immunoglobulin-like (Ig-like) domains that form the extracellular region, a stem, a transmembrane region, and a cytoplasmic region. The different members of the KIR family are named according to the number of extracellular domains (2D or 3D), the length of the cytoplasmic tail [long (L) or short (S)], followed by a number to distinguish different members having the same structure but differing in amino acid sequence, for example, KIR3DL1, KIR3DL2 (Figure 5.1). In addition, they are grouped based on the presence or absence of a charged amino acid in the transmembrane region [17]. KIR receptors are further subclassified into three groups, types I–III, according to the configuration of their Ig-like domains. Type I receptors utilize a D1 and D2 domain and lack the D0 (membrane

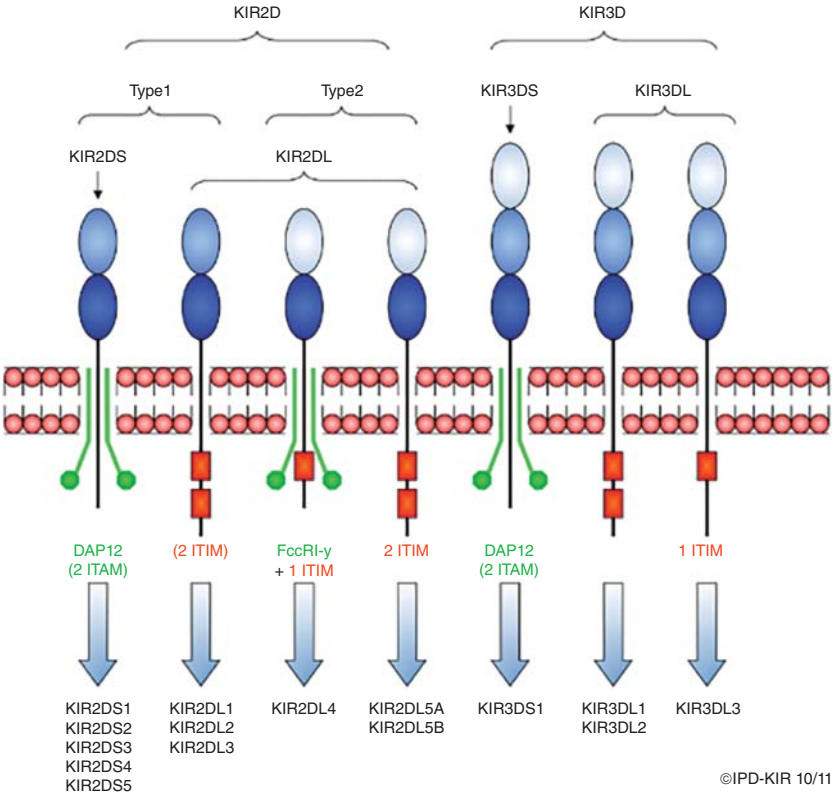

Figure 5.1 KIR protein structures. The structural characteristics of two and three Ig-like domain KIR proteins are shown. The association of activating KIR to adaptor molecules is shown in green, whereas the ITIM of inhibitory KIR are shown as red boxes. Source: EMBL/ permission of Dr James Robinson, Anthony Nolan Research Institute, London, United Kingdom (http://www.ebi.ac.uk/ipd/kir/).

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distal) domain. The 3DL and 3DS KIR genes contain three Ig-like domains, D0 (membrane-distal), D1 (middle), and D2 (membrane-proximal). The 2DL and 2DS KIR genes, with the exception of 2DL4 and 2DL5, lack the D0 domain. The 2DL4 and 2DL5 genes contain extracellular domains D0 and D2 [18, 19]. The extracellular domains contain the binding site for the HLA ligand [20]. NK cell inhibitory and activating receptors are characterized by high homology within the extracellular portion of the receptor but differ within the transmembrane and intracytoplasmic domains [21]. KIR can be classified into two groups according to the size of the cytoplasmic tail: those having long cytoplasmic tails—inhibitory KIR; and those having short cytoplasmic tails—activating KIR. Inhibitory KIR contains two copies of an immune tyrosine-based inhibitory motif (ITIM) in the intracytoplasmic domain [22]. ITIMs are structurally made up of a tyrosine residue, an N-terminal isoleucine, valine, leucine, or serine, and C-terminal leucine or valine (I/V/L/SxYxxL/V). The interaction between the receptor and its MHC ligand results in the phosphorylation of the tyrosines in the ITIM units and recruitment of the protein tyrosine phosphatase, SHP-1 or SHP-2. This results in the inhibition of NK cell activation [17, 22, 23]. Activating receptors contain a charged amino acid residue in the transmembrane domain and lack an ITIM in the intracytoplasmic domain [22]. The presence of a charged amino acid residue in the transmembrane domain of activating receptors enables them to bind noncovalently with specialized signal-transducing polypeptides DAP-12 and DAP-10, known as adaptor molecules. DAP-12 and DAP-10 are disulfide-linked tyrosine phosphorylated homodimers containing an immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic domain. The activating receptor/adaptor molecule complexes in turn activate protein tyrosine kinases such as Syk or ZAP-70, which result in cellular activation [17, 22, 24].

5.5.2 KIR Genomic Organization

Sequencing of the LRC region containing the KIR gene family on human chromosome 19q13.42 identified 16 highly conserved, tightly clustered KIR genes and pseudogenes, arranged in head-to-tail orientation [25]. All KIR genes share similar exon–intron structure of nine exons and eight introns, where each gene is separated by approximately 2.4 kb intergenic sequence. The one exception is the region between 3DP1 and 2DL4, which is approximately 14 kb. The KIR gene sequences, including intergenic regions, are closely related and are likely to have evolved recently [26]. Three framework genes found in all individuals have been identified: 3DL3 at the centromeric end, 2DL4 in the middle, and 3DL2 at the telomeric end. All three genes are located near the unique sequences that may prevent loss of these genes by DNA looping [27]. All KIR genes have the AG-GT exon–intron splice signal, except for exon 3 of 2DL1, which has CG splice site. Exons 1 and 2 encode the signal peptide, exons 3–5 encode the Ig-like domains (D0, D1, and D2 respectively), exon 6 encodes the stem connecting the D2 domain to the transmembrane region that is encoded by exon 7, and exons 8 and 9 encode the cytoplasmic tail. In the type 1 2DL/S KIR genes, exon 3 is a pseudoexon that is not expressed due to exon

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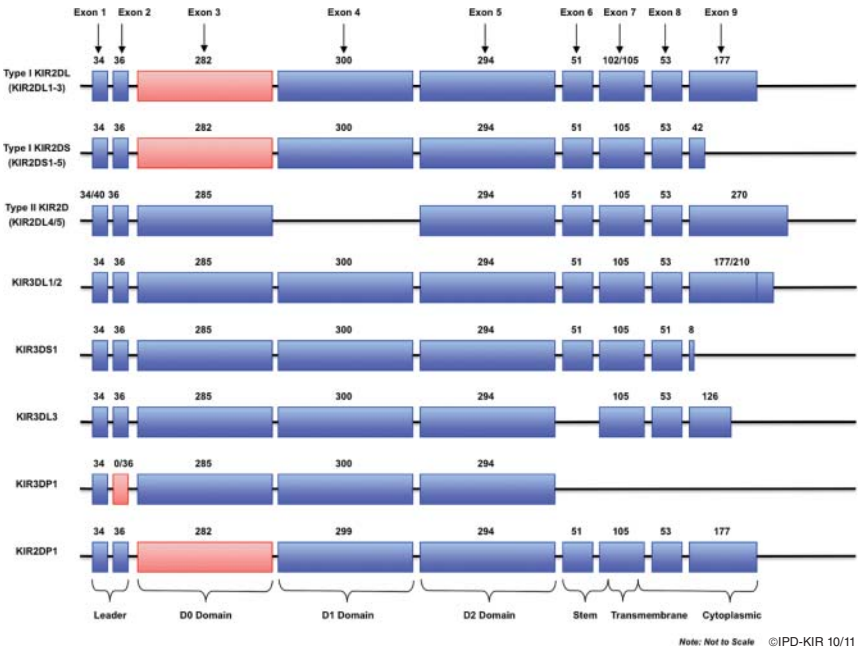

Figure 5.2 Genome organization of KIR. The coding regions of the exons are represented as blue boxes; their size in base pairs is shown in digits above them. The pseudoexon 3 and the deleted KIR3DP1 exon 2 are shown in red. The brackets at the bottom of the diagram illustrate the way in which the exons code for each protein domain and region.

skipping. In the type II 2DL KIR genes, the region corresponding to exon 4 in all other KIR genes is deleted. The KIR3D genes have all nine exons. The most striking difference between the KIR genes is the length, ranging from 3.9 to 16.3 kb. This variation is primarily due to insertions/deletions of retroelements in the introns of these genes [26]. The greatest number of nonsynonymous substitutions is seen in exons 3–5, which encode the D0, D1, and D2 domains, respectively. This variation is most likely due to selective pressure operating on the interaction of these domains with different MHC class I molecules (Figure 5.2).

5.5.3 KIR Nomenclature

The naming of KIR genes is the responsibility of the HUGO (Human Genome Organisation) Genome Nomenclature Committee. The names given to the KIR genes are based on the structures of the molecules they encode. The first digit following the KIR acronym corresponds to the number of Ig-like domains in the molecule, and the "D" denotes "domain." The D is followed by either an "L," indicating a "long" cytoplasmic tail, an "S," indicating a "short" cytoplasmic tail, or a "P," for pseudogenes. The final digit indicates the number of gene encoding a protein with this structure [28]. Thus, KIR2DL1 has two Ig-like domains and a long cytoplasmic tail. The naming of the KIR genes includes an asterisk (separator) after the gene name before a numerical allele designation. The first three digits of the numerical designation

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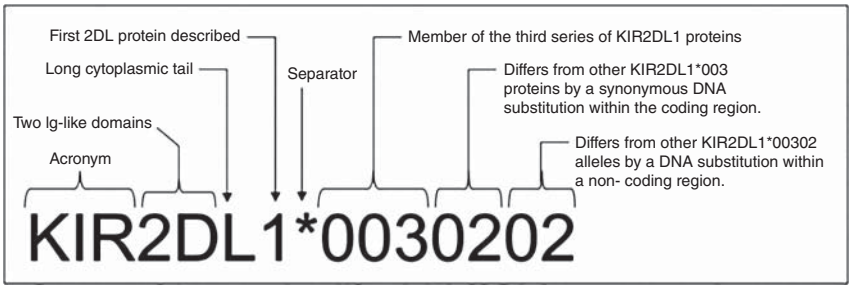

Figure 5.3 KIR nomenclature. Source: EMBL/permission of Dr James Robinson, Anthony Nolan Research Institute, London, United Kingdom (http://www.ebi.ac.uk/ipd/kir/).

are used to indicate alleles that differ in the sequences of their encoded proteins. The next two digits are used to distinguish alleles that only differ by synonymous (noncoding) differences within the coding sequence. The final two digits are used to distinguish alleles that only differ by substitutions in an intron, promoter, or other noncoding region of the sequence [29] (Figure 5.3).

5.5.4 KIR Haplotypes

Diversity is an important characteristic of the human KIR gene system. Diversity of the KIR gene family includes differences in sequence differences of the KIR genes (allelic polymorphism), expression of the KIR genes, and the KIR genes inherited. Uhrberg et al. [30] first proposed two broad distinct KIR haplotype groups referred to as KIR A and B haplotypes. This classification was based on the differences in the number of KIR genes present in 68 blood donors and the absence (haplotype A) or presence (haplotype B) of a 24 kb HindIII fragment on Southern blotting. Subsequent analysis by Witt et al. [31] of linkage disequilibrium (LD) between KIR sequences obtained in a panel of 147 individuals also showed that the KIR genes are divided into two distinct haplotypes based on the presence or absence of KIR2DL1 and KIR2DL3 (KIR A haplotypes) or KIR2DL2 (KIR B haplotypes). It subsequently became clear that the approach of Witt et al. [31] was based on genes at the telomeric end of the KIR complex, whereas the HindIII fragment resides in the centromeric end of the complex. The two approaches, however, resulted in a very similar classification of haplotypes due to the extensive LD between the KIR genes. The KIR A and B haplotype consists of four framework genes—3DL3, 2DL4, 3DP1, and 3DL2—with very few exceptions, and are present on all haplotypes. The A haplotype is generally nonvariable and is composed of four inhibitory receptors—2DL1, 2DL3, 3DL1, and 3DL2—the activating receptor 2DS4, and pseudogene KIR2DP1. The B haplotype is more variable and has greater gene content and is composed of the inhibitory receptors 2DL1, 2DL2, 2DL5, and the four activating receptors 2DS1, 2DS2, 2DS3, and 3DS1. Diversity in the A haplotype is due to allelic polymorphism while the B haplotype is both polymorphic and polygeneic. Hsu et al. reported on specific haplotypes within 12 families and presented a model for the genomic organization of the human KIR region (Figure 5.4) [32]. Hsu et al. also identified a new variant of 2DS4 referred

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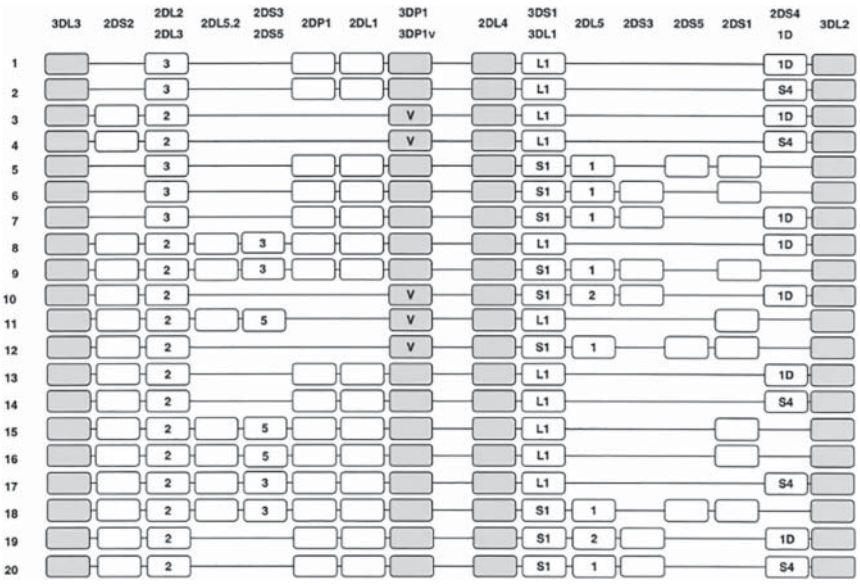

Figure 5.4 KIR haplotypes defined by family studies and genomic sequencing. Allele designations for KIR2DL5 are shown within the corresponding box. 1 collectively denotes the expressed 2DL5 variants 2DL5.1 and 2DL5.3 and 2 collectively denotes the nonexpressed 2DL5 variants 2Dl5.2 and 2DL5.4. Shaded boxes denote framework KIR genes.

to as KIR1D (now known as 2DS4*0030101) characterized by a 22 base pair deletion in the second extracellular Ig-like domain, predicted to encode a soluble protein with one intact Ig-like domain and not likely to fulfill the same functions as the intact 2DS4. Typing for KIR1D revealed that the previously defined haplotype A could be further divided into two groups: one containing 2DS4 and the other the inactive KIR1D variant. The haplotype containing the inactive KIR1D allele appeared more common, comprising 73% of KIR A haplotypes and a frequency of 39% in the Caucasian population. The haplotype containing 2DS4 had a haplotype frequency of 12%. This combined haplotype frequency of both KIR A haplotype subgroups in this study was 51%, consistent with previous reports [30, 31].

LD analysis undertaken by Hsu et al. [32] supported the findings of Witt et al. [31]. Hsu et al. reported that several pairs of KIR genes and pseudogenes display negative LD, implying they could be alleles; KIR2DL3 and KIR2DL2, KIR3DS1 and KIR3DL1, KIR2DS3 and KIR2DS5, KIR2DS1 and KIR2DS4 and KIR1D. While others have positive LD, implying proximity along the chromosome and co-segregation, for example, KIR2DL2 and KIR2DS2. Hsu et al. also proposed a KIR haplotype model consisting of two halves. The first a centromeric half bordered upstream by KIR3DL3 and comprised of KIR genes upstream of KIR2DL4 and the second a telomeric half bordered downstream by 3DL2 and comprised of KIR genes downstream of KIR2DL4 [32]. The centromeric part is characterized by the presence of KIR2DL3 or KIR2DL2 but not both and extremely rarely neither. Where KIR2DL2 is present, it is always paired with KIR2DS2. KIR2DL3 defines a partial

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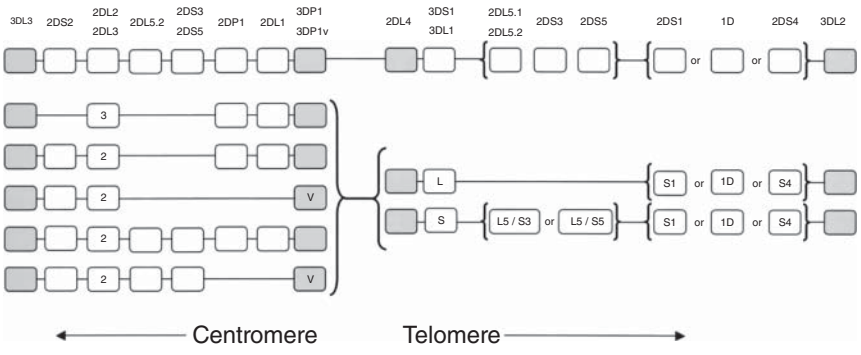

Figure 5.5 KIR haplotype model. Source: Hsu et al. [32]/permission of John Wiley & Sons.

haplotype and is always paired with KIR2DP1, -2DL1, and -3DP1. In contrast, while KIR2DS2 and 2DL2 can also be found with 2DP1, -2DL1, and -3DP1, 2DS2, and 2DL2 define a separate partial haplotype, which is characterized by the presence of the exon 2-containing pseudogene variant 3DP1v and by the absence of 2DP1 and 2DL1. The unexpressed 2DL5 variant, when seen in the centromeric half, is paired with 2DL2 and 2DS3 or rarely with 2DL2 and 2DS5. The telomeric half downstream of KIR2DL4 is characterized by the presence of KIR3DL1 or KIR3DS1 but not both, or, rarely, neither. The presence of KIR3DL1 indicates a "short" telomeric segment with either KIR2D4 or KIR1D followed by KIR3DL2. The presence of KIR3DS1 defines a: "long" telomeric segment containing KIR2DL5 subtype with either KIR2DS3 or KIR2DS5 followed by a locus occupied by either KIR2DS1, KIR2DS4, or KIR1D and completed with KIR3DL2 (Figure 5.5). Furthermore, the sequencing of complete KIR haplotypes of 12 selected cell lines by Pyo et al. identified that the seven gene content KIR haplotypes are all combinations of four centromeric and two telomeric gene motifs [33]. Further diversification of KIR haplotypes is at the allele level.

5.5.5 KIR Ligands

Inhibitory KIR receptors interact with the classical class I molecules, HLA-A, -B, and -C, to inhibit NK cell-mediated lysis. KIR recognition is not only locus-specific but also specific for certain groups of alleles sharing a common epitope. Type I KIR2D receptors interact with HLA-C alleles, while type III KIR3D receptors interact with either HLA-A or -B alleles bearing the Bw4 epitope (KIR3DL1) or with the A3/11 epitope carried by HLA-A*03 and HLA-A*11 allotypes (KIR3DL2) [34]. These receptors do not interact with Bw6-associated HLA-B alleles. HLA-C alleles can also be classified into two groups based on a dimorphism at positions 77 and 80 in the α1 domain. Based on this dimorphism, HLA-C alleles can either have Asn77/Lys80 (C2 epitope) or Ser77/Asn80 (C1 epitope). Those having the C2 epitope include Cw2, Cw4, Cw5, Cw6, Cw1602, Cw17, and Cw18. Those having the C1 epitope include Cw1, Cw3, Cw7, Cw8, Cw13, Cw14, and Cw1601 [5, 35]. The inhibitory receptor KIR2DL1 and the activating receptor KIR2DS1 recognize HLA-C alleles having

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the C2 epitope, whereas the inhibitory receptors KIR2DL2 and KIR2DL3 and the activating receptor KIR2DS2 recognize those having the C1 epitope. However, the binding affinity of HLA-C with the KIR2DS activating receptors is weaker than for the inhibitory receptors. It may be possible that the affinity of activating receptors for HLA-C is lower than that of inhibitory receptors to ensure that inhibition overrides activation, yet it is sufficient to activate NK cells in the absence of an inhibitory signal [36, 37].

5.5.6 KIR Receptor Expression

Human NK clones express different numbers and combinations of KIR receptors [38]. The only requirement for each NK clone with lytic activity is that it expresses at least one inhibitory receptor with specificity for a self-class I MHC molecule. The inhibitory receptor can belong to either the KIR or CD94-NGG2 family. The range of different KIR receptors expressed by the repertoire of NK clones in an individual is primarily determined by the KIR genotype, but the individual HLA genotype determines which KIR can be expressed as the only inhibitory receptor on individual NK cell clones [30]. Receptor selection occurs during NK cell development to ensure only NK cells with an inhibitory receptor for a self-ligand are permitted to become armed for cytotoxicity. It was thought that lytic NK cells without an inhibitory receptor for self-HLA class I do not appear in peripheral circulation [30, 38]; however, such cells, though rare, can be identified but appear do not seem to be "armed" for cytotoxicity. It is thought that arming requires interaction between the inhibitory receptor and its ligand [39]. This group showed that NK cells are "licensed" for functional competence by interactions between inhibitory NK cell receptor and a self MHC class I molecule, a process known as licensing. In mouse models, the authors showed that in a large number of NK cells from wild-type mice cytoplasmic IFN-γ could be produced but NK cells from mice deficient in MHC class I molecules could not do so. This study defined a mechanism for NK cell tolerance, NK cells that express a self-MHC-class I specific inhibitory receptor become licensed for functional competence while those lacking such a receptor do not become licensed and are functionally inert.

5.5.7 NK Cell Allorecognition

Although KIR receptors determine the allospecificity of NK cells, the co-expression of CD94-NKG2A, which mediates inhibition when engaging the nonpolymorphic HLA-E molecule, renders an NK cell nonalloreactive toward targets expressing HLA-E. To define the rules governing NK cell self-tolerance and alloreactivity, Valiante and colleagues studied the expression of KIR and CD94-NKG2A receptors on NK clones obtained from two blood donors PP and NV [38]. The two donors differed in their class I HLA and KIR genes. Both donors were heterozygous at HLA-A, -B, and -C and shared one class I HLA haplotype. Donor PP's HLA type included only one HLA ligand for inhibitory KIR (C1), whereas donor NV's HLA

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type included all three ligands (C1, C2, and Bw4). NK clones from both donors were shown to lyse the class I HLA-deficient 721.221 cell line but not their autologous B cell lines. 721.221 cells transfected with a single-class I HLA allele were lysed to varying degrees but in a reproducible manner by the different NK clones. They showed that every cytotoxic NK cell expressed either an inhibitory KIR that recognized a self-class I HLA epitope or CD94:NKG2A. Using antibody-blocking experiments, the authors showed that the class I HLA-mediated inhibition of NK cell clones for donor PP, whose HLA type included only the C1 epitope, was accounted for by two receptors, KIR2DL3 and CD94-NKG2A. NK clones expressing KIR2DL3 were strongly inhibited by autologous HLA allotypes belonging to the C1 group (C*03:04 and *07:02). Donor NV's HLA type included all three HLA epitopes, and the inhibitory KIR specific for each HLA-C epitope was individually or co-expressed on some 30–50% of the peripheral blood NK cells while KIR3DL1, the receptor for NV's Bw4 ligand (B*27:08), was expressed on very few NK cells. KIR2DL1 was shown to have specificity for HLA-C alleles belonging to the C2 group and KIR2DL2 specificity for the C1 group. All other inhibition not mediated through these receptors could be accounted for by CD94-NKG2A.

The extent to which NK cell clones from one donor are inhibited by the HLA ligands of another donor was also investigated. Some NK clones from donor NV (C1+, C2+, Bw4+) lysed targets from PP (C1+ only), whereas PP did not have any clones that could lyse targets from donor NV. The NK alloreactivity demonstrated could be explained by the repertoire of receptors and the HLA ligands expressed by the two donors. KIR2DL3 and CD94-NKG2A were the dominant inhibitory receptors in donor PP, and since NV's cells had HLA ligands for both of these receptors there was no NK alloreactivity of donor PP's cells toward NV cells. In contrast, NV had a subset of NK cells that used KIR2DL1 as their only inhibitory receptor, and since PP's cells did not express the ligand for this receptor (C2), these NK cells killed PP targets. Thus, the general rule emerged that NK cells from individual A will be alloreactive to cells from individual B if individual B lacks a KIR ligand expressed by individual A. Individual A's NK cells will be tolerant of cells from individual B if individual B has the same or additional KIR ligands as individual A [38].

5.5.8 NK Alloreactivity and Hematopoietic Stem Cell Transplantation

Ruggeri et al. [40, 41] showed that donor-versus-recipient NK cell alloreactivity resulted in less relapse, rejection, graft-versus-host disease (GVHD), and increased survival in human haploidentical transplants and murine transplant models. Most importantly in haploidentical transplants, NK cells were shown to mediate GVL effects without GVHD. Since then, there have been several studies showing either no effect or beneficial effects of NK alloreactivity based on different NK cell alloreactivity models in different transplantation settings (reviewed in Hadjis and McCurdy [42]). Several models have been proposed to predict KIR incompatibility between recipient and donor post transplantation. Each model is based on whether the mismatches are based around the KIR receptors or the KIR ligands. The five models include the ligand–ligand mismatch model, when the recipient lacks a

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ligand that is present in the donor [41]; receptor–ligand/missing-ligand model, if the KIR of the donor does not recognize the MHC molecule of the recipient there will be alloreactivity [43, 44]; receptor–receptor mismatch/haplotype model, donors with KIR B haplotypes have been shown to have improved outcomes, which has been postulated to be due to more activating KIR genes [45]; missing licensing model, when an NK cell that has been "licensed" or "educated" based on a self-specific inhibitory receptor to a specific self MHC class I ligand encounters a recipient cell that does not have the same self MHC class I ligand, there is alloreactivity [46]; and NK cell genotyping, mismatches of KIR genotypes result in alloreactivity [47]. While some studies have shown an association with NK alloreactivity and decreased leukemia relapse, it is yet to be formally implemented routinely. Further studies are required to understand how to enhance NK alloreactivity, especially under newer treatment regimens using post-transplant cyclophosphamide.

5.5.9 KIR and Infections

NK cells are involved in the early stages of the innate immune response against viral infections by producing cytokines and causing cytotoxicity. The activating KIR allele, KIR3DS1, in combination with HLA-B alleles that encode molecules with isoleucine at position 80 (HLA-B Bw4-80I) is associated with delayed AIDS progression in individuals infected with human immunodeficiency virus type 1 (HIV-1). In the absence of KIR3DS1, the HLA-B Bw4-80Ile allele was not associated with any of the AIDS outcomes measured in this study; however in the absence of HLA-B Bw4-80Ile alleles, KIR3DS1 was significantly associated with more rapid progression to AIDS [48]. Additionally, a variation in surface expression of both receptor and ligand as well as differential binding capacity of specific KIR3DL1 allotypes to certain HLA-Bw4 ligands has been shown to be associated with HIV progression. Boudreau et al. showed that high cell surface expression of both receptor and ligand, as well as strong binding between KIR3DL1 and HLA-Bw4, cooperatively generate the most potent reactivity of primary NK cells against HLA-negative target cells and autologous $CD4^+$ cells infected with HIV [47]. More recently, a nonsynonymous variant in the KIR3DL1 gene (I47V) that modifies B*57 protection against HIV-1 has been described [49]. The protective effect of I47V was restricted to the B*57:01 subtype, and no such effect was observed for the closely related B*57:03 suggesting that differences in positioning or sequence of peptides bound to B*57 are in part responsible for the protective effect of KIR3DL1 47V. Together, these studies highlight the importance of synergistic effects of HLA and KIR ligands on the control of the disease. Beneficial effects of KIR2DL3 and HLA-C group 1 ligands following exposure to hepatitis C virus have also been demonstrated [50, 51]. Both studies showed the KIR2DL3-C1 homozygotes were more common in sustained virological response, and most of this association was due to the C1-Cw*03 variant. It was proposed that as the KIR2DL3 tags haplotype A, which contains fewer activating KIR genes, this association was consistent with insufficient activation of NK cells in the context of HLA-C2C2 inhibition as the basis for increased risk of treatment failure.

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5.6 CD94-NKG2 Receptors

The CD94-NKG2 receptors are dimeric proteins consisting of an invariant CD94 polypeptide disulfide linked to a member of the NKG2 family of receptors [12]. The CD94 and NKG2 polypeptides are C-type lectin receptors, the genes for which are found closely linked on chromosome 12 in the NKC [12, 52, 53]. While a single gene encodes CD94, there are five NKG2 genes: NKG2A/B, --2C, -2E, -2F, and -2D [54–57] (Figure 5.6). Despite having a common name, the NKG2D receptor is quite distinct from the other receptors of the NKG2 family. Co-immunoprecipitation and transfection experiments showed that CD94 associated with NKG2A and -2C but not with NKG2D [54, 57]. NKG2D significantly differs in sequence from the other NKG2 receptors, and rather than dimerizing with CD94, is expressed as a homodimeric receptor. Moreover, NKG2D interacts with stress-induced ligands, notably MHC class-I-chain-related protein (MIC)-A, MIC-B, and ULBP in humans.

The CD94 polypeptide is a 30 kD protein that lacks a cytoplasmic region, and the signaling motifs associated with transducing activating or inhibitory signals, and the signaling capacity of the CD94-NKG2 receptors is driven by the NKG2 subunit. NKG2A and its alternatively spliced variant NKG2B possess cytoplasmic ITIM motifs that recruit SHP-1, which are characteristic of inhibitory receptors [56, 59]. On the other hand, NKG2C and NKG2E (and its splice variant NKG2H) lack ITIMs but possess an a positively charged arginine residue in their transmembrane region, which, like activating forms of KIR, facilitates the association with the ITAM-containing adapter molecule DAP12 that is crucial for signal transduction [24]. Therefore, the CD94-NKG2 family includes members of both activating and inhibitory capability.

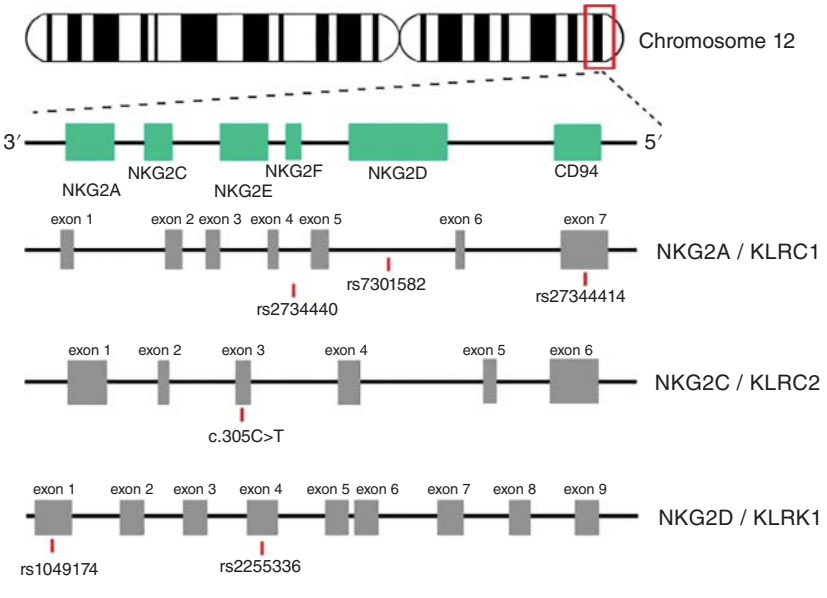

Figure 5.6 Genomic organization of the C-type lectin receptors on human chromosome 12. Source: Siemaszko et al. [58]/Springer Nature/CC BY 4.0.

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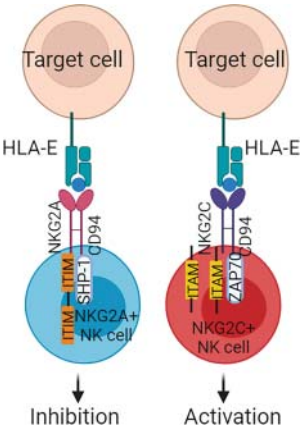

Figure 5.7 HLA-E is the ligand for inhibitory CD94-NKG2A and activating CD94-NKG2C receptors. Source: Created with BioRender.com.

These receptors were originally described as being expressed on NK cells; however, they are also expressed on subsets of $CD8^+$, $CD4^+$, and γδ T cells [60–62].

CD94-NKG2 Ligand Interactions: The ligand for CD94-NKG2A and CD94-NKG2C receptors is the nonclassical MHC molecule HLA-E [63–65] (Figure 5.7). Unlike classical HLA, HLA-E displays limited polymorphism and is broadly expressed on the surface of most cells at low levels. HLA-E exists as two common alleles, HLA-E*01:01 and E*01:03, with E*01:03 being expressed at higher levels on the cell surface [66].

The molecular details of the interaction between HLA-E and CD94-NKG2A have been described. While the signaling capacity stems from the NKG2 polypeptide [54, 67], the CD94 chain plays the dominant role in the receptor/ligand interaction [68, 69]. Structural and mutagenesis studies of CD94-NKG2A and HLA-E found that the CD94 subunit is positioned largely over the α1 subunit of HLA-E while the NKG2A is over the α2 helix [69, 70]. Surprisingly, NKG2A is somewhat peripheral to the interaction, with mutagenesis studies suggesting that few residues in NKG2A were energetically important for binding to HLA-E [69] and the structure of the receptor/ligand complex similarly showing that NKG2A contacts only seven residues on HLA-E [68] (Figure 5.8).

As yet, there is no structural data describing the interaction of HLA-E with CD94-NKG2C. Despite 92% homology in the extracellular domain between CD94-NKG2A and CD94-NKG2C, there is a six-to-eight-fold increase in the affinity of CD94-NKG2A for HLA-E compared with that of CD94-NKG2C [65, 69, 71]. Mutagenesis studies suggest that this affinity difference is attributable to conformational difference in the loop at the C-terminus of the α-helix in the lectin domains of the NKG2 subunit, possibly impacted on the dimer interface or the conformation of CD94 [69]. Moreover, CD94-NKG2C appears to be more sensitive to the peptide bound to HLA-E as the same HLA-E/peptide complex is capable of inhibition of NKG2A-expressing NK cells, but not activation of NKG2C-expressing NK cells [72].

Optimal cell surface expression of HLA-E requires it to bind a nonamer peptide derived from the leader sequence of MHC class I molecules [73]. This peptide is

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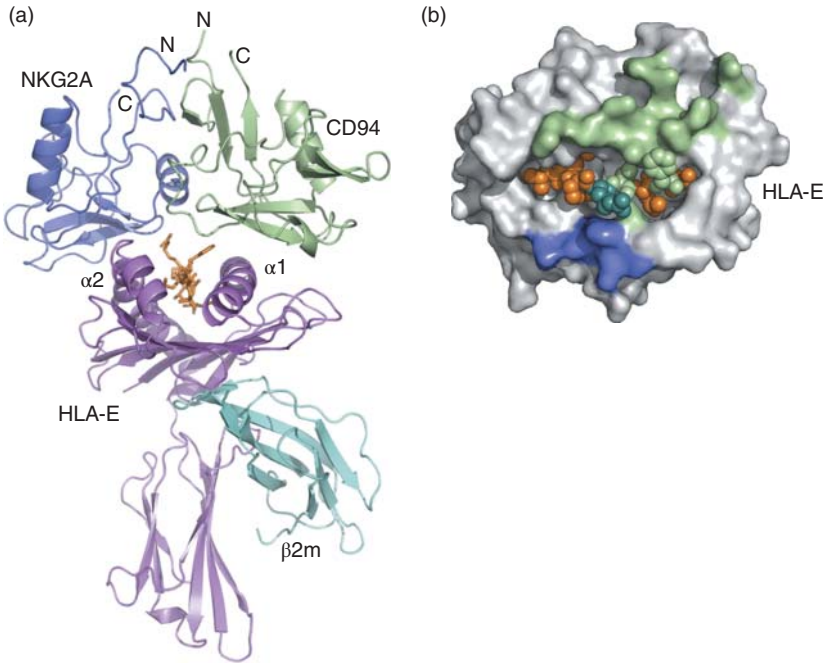

Figure 5.8 (a) Overview of the CD94-NKG2A receptor docking onto HLA-E. (b) Comparison of the CD94 footprint (green) with NKG2A footprint (blue) on HLA-E. Source: Adapted from Petrie et al. [68].

released into the cytosol where it is transported to the endoplasmic reticulum via the transporter associated with antigen processing (TAP) complex and then binds to HLA-E [74]. Early studies identified that the HLA-E/CD94-NKG2A interaction on NK cells was in line with the classical NK cell "missing self" hypothesis [10]. That is, in healthy cells that express normal levels of HLA and that have intact antigen presentation pathways, HLA-E is expressed at the cell surface in complex with HLA-derived peptides, where it can interact with CD94-NKG2A. Upon binding of HLA-E, the ITIMs of NKG2A are phosphorylated, resulting in the recruitment of intracellular phosphatases (SHP-1), thereby suppressing NK cell activation. In contrast, when classical HLA is lost due to pathogen infection or transformation, HLA-E is not expressed at the cell surface, potentially allowing for NK cell activating signals to dominate. However, subsequent studies have shown the activation and inhibition of NK cells is a highly complex, multifactorial process. For example, the sequence of the HLA-E-bound peptide impacts on the interaction with CD94-NKG2 receptors. Functional studies using NK cell clones expressing either CD94-NKG2A or CD94-NKG2C showed that the peptide corresponding to the leader peptide derived from HLA-G but not the analogous sequences in classical class I molecules preferentially activated NK cell clones expressing NKG2C [75]. Similarly, direct measurements of the affinity of the interaction between CD94-NKG2 receptors and HLA-E showed that when bound to the peptide derived from HLA-G, HLA-E interacted

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with both CD94-NKG2A and CD94-NKG2C with higher affinity than when bound to the peptides derived from classical HLA class I molecules [65, 76, 77].

There is also polymorphism in the leader-derived peptides from classical HLA, and peptides derived from certain HLA-B alleles bind poorly to HLA-E [65]. HLA-B alleles are divided into two groups, based on the presence of a threonine (T) or a methionine (M) at position -21 in the leader sequence peptide, with leader sequence peptides containing T binding poorly to HLA-E. The -21 HLA-B dimorphism also plays an important role in NK cell "education," a process by which NK cells become functionally active through the interaction between inhibitory receptors and self-HLA, thereby preventing self-reactivity. Individuals of an MT or MM genotype are reported to have NK cells with increased potency, presumably because of differential impacts on NKG2A-driven NK cell education [78] (Figure 5.9).

Another peptide that is proposed to manipulate NK cell function through HLA-E is derived from human CMV. CMV is a ubiquitous virus belonging to the Herpesviridae family, which creates a lifelong largely asymptomatic infection in immunocompetent individuals. However, CMV is a significant cause of morbidity and mortality in immunocompromised individuals, such as those with HIV infection or following transplantation [79]. The CMV protein UL40 contains a sequence that is homologous to the leader sequence in most HLA-C molecules [80]. Critically, the UL40-encoded mimic was shown to bind HLA-E via a TAP-independent mechanism [80, 81]. Consequently, following CMV infection and TAP blockade, cell surface expression of HLA-E can be maintained via the expression of UL40, allowing for infected cells to avoid NK cell lysis through CD94-NKG2A inhibition [80, 81]. However, the region of UL40 that encodes the HLA-E binding peptide is also polymorphic, and certain variants reduce the affinity between HLA-E and both CD94-NKG2A and -NKG2C [72].

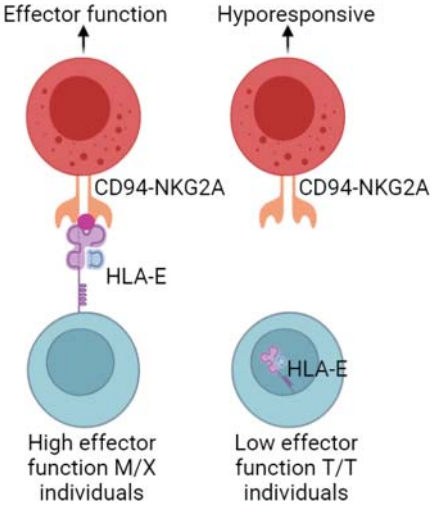

Figure 5.9 CD94-NKG2A expressing NK cells from TT individuals are hyporesponsive. Source: Created with BioRender.

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5.6.1 Role of CD94-NKG2 Receptors in CMV Infection

There is a body of literature indicating that CMV infection influences the NK cell repertoire and in particular the expression of CD94-NKG2 receptors [82–84]. For example, a study of the proportions of CD94-NKG2A and CD94-NKG2C from peripheral blood mononuclear cells showed that positive CMV serology was strongly associated with higher proportions of CD94-NKG2C expressing NK cells [83]. Similarly, co-culture of NK cells with CMV-infected fibroblasts results in the expansion of CD94-NKG2C-positive NK cells [82]. Various studies have linked the expansion of NKG2C+ NK cells with CMV replication in immunocompromised transplant recipients. CMV replication drives the sustained expansion of this subset following hematopoietic stem cell transplantation (HSCT), which appears to persist in the absence of active viral replication [85, 86]. There is evidence this subset is actively involved in the control of CMV replication as higher proportions have been associated with lower viral loads following HSCT [87], kidney [88], and lung [89] transplantation.

Subsequent studies have characterized the CMV-driven NKG2C+ NK cell subset to be a polyfunctional, highly cytotoxic phenotype, marked by the absence of NKG2A and the expression of CD57. These CMV-expanded NKG2C+ NK cells demonstrated a heightened capacity to respond to subsequent exposure to CMV and have therefore been termed memory or adaptive NK cells [85, 86]. Intriguingly, the expansion of NKG2C+ NK cells appears independent of the CMV UL40 protein as a deletion mutant lacking UL40 stimulated the expansion of CD94-NKG2C-positive NK cells [84]. The identification of the peptide bound to HLA-E recognized by NKG2C+ NK cells that drive the expansion of this subset remains elusive. The peptide may be of CMV origin, or alternatively, as CMV also drives the upregulation of HLA-G [90], it may be that the increased availability of the HLA-G leader peptide also promotes upregulation of HLA-E surface expression. Regardless, CMV infection appears to have opposing effects on different NK cell subsets by suppressing activation through NKG2A via UL40, while concurrently promoting activation of NKG2C+ expressing NK cells (Figure 5.10).

5.6.2 CD94-NKG2 Receptors in Infection and Malignancies

The expansion and/or heightened function of NKG2C+ NK cells have been demonstrated in non-CMV viral infections, including Hantavirus [91], Epstein–Barr virus [92], hepatitis [93], chikungunya [94], HIV [95], and SARS-CoV-2 [96], although CMV co-infection appears to be the common feature of many of these observations. NK cells have been strongly linked to the control of HIV infection, most notably with certain KIR/HLA combinations being associated with disease progression [97]. However, a study by Jost et al. suggested that HLA-E binds HIV- and influenza-derived peptides to directly activate NKG2C+ NK cells [98]. Furthermore, structural studies indicate that the peptide-binding groove of HLA-E may tolerate more diverse peptides than originally believed by virtue of the peptide being able to adopt alternative conformations in the peptide-binding groove [99].

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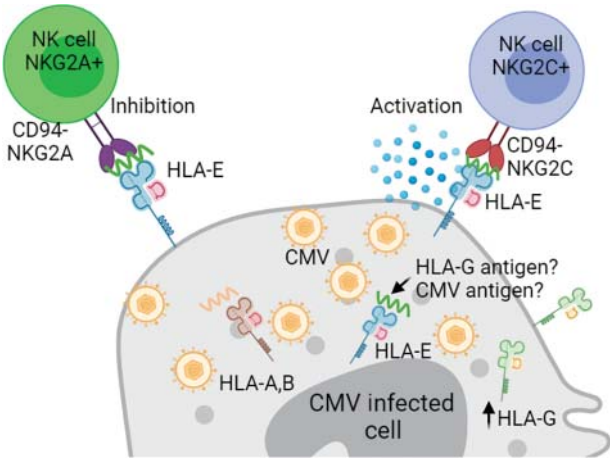

Figure 5.10 CMV has opposing effects on different NK cell subsets. Source: Created with BioRender.com.

To this end, HLA-E has been reported to bind viral peptides derived from HIV [100], influenza [98], Epstein–Barr birus [101], and hepatitis B [102] and -C [103]. However, while some of these viral peptide/HLA-E complexes may not be recognized by CD94-NKG2A, thereby triggering NK cell function via missing self, several pathogens may harness this axis as an immune evasion mechanism. For example, a high proportion of lymphocytes expressing NKG2A were found in patients with active COVID-19 compared to convalescent individuals, and these NKG2A-expressing cells have reduced the capacity to degranulate and produce cytokines [104].

Unlike the expression of classical HLA, the expression of HLA-E is maintained or often upregulated in solid tumors and hematopoietic malignancies and, in addition, NKG2A expression is reportedly overexpressed in tumor-infiltrating cells (TILs). Higher levels of HLA-E and NKG2A are both associated with poorer outcomes, presumably due to the failure to clear the malignancy as the infiltrating TILS are inhibited (reviewed in [105]). This has prompted several groups to investigate NKG2A blockade as potential immunotherapy for cancer patients. However, to date, studies have shown that anti-NKG2A monoclonal antibodies alone are not effective for tumor clearance. For example, the NKG2A blocker monalizumab, although well tolerated, was not useful as a single therapy in gynecological [106] or head and neck [107] malignancies. However, there are now several trials underway using monalizumab as combination therapy with anti-PDL1 inhibitors that are showing more promising results [105].

The ability of HLA-E to present HLA-B leader peptides may also be important in outcomes following hematopoietic malignancies. Solomon et al. found that following HSCT for lymphoid malignancies, recipients with Met in the B-leader showed improved overall survival and lower relapse of disease [108]. Similarly, Hallner et al. found improved disease-free and overall survival in patients with Met containing B-leader sequences after immunotherapy for AML [109]. Although there

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has been no mechanistic explanation for these observations, the likely explanation is the ability of HLA-E to interact with CD94-NKG2 receptors expressed on NK or T cells, which would influence not only their effector function but also the "education" of NK cells following immune reconstitution.

The Petersdorf group has also described the impact of mismatching donor and recipient B-leader on the outcomes following HSCT. Intriguingly, among recipients with a single HLA-B allele mismatch with their donor, acute GVHD was higher in HLA-B-leader mismatched recipients [110]. Furthermore, GVHD was higher with recipients that shared Met B-leader allotype with the donor. This group subsequently developed an HLA-B leader tool for the assessment of donor–recipient B-leader mismatching, which could be used for donor selection for HSCT recipients when finding an HLA-matched donor was proving difficult [111]. It is hoped that future studies will not only confirm the clinical utility of the B-leader tool in donor selection, but also uncover the mechanism(s) behind the impact of the HLA-B leader dimorphism on clinical outcomes. In conclusion, the CD94-NKG2 receptors are important in NK cell education and play a pivotal role in controlling viral infection and outcomes following solid tumor and hematological malignancies. Exploiting this axis for future therapies will require a deeper understanding of the molecular processes involved to fully harness their potential.

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6

The HPA System

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6.1 Platelet Antigens

Platelets share antigens with other blood cells such as the human leukocyte antigens (HLA)-A, -B, and -C and the red blood cell antigens: ABH, Lewis, I, i, and P.

The expression of group A and group B antigens on platelets corresponds with the ABO blood group. Individuals with blood groups A and B express H and A or H and B antigens, respectively, on the platelets. Group O individuals express only H antigens on their platelets. ABH antigens are carried on various glycoproteins expressed by platelets, especially on GPIIb (CD41) and PECAM (CD31). The number of A and B antigens on platelets is genetically determined and varies between individuals. Next to these antigens shared with red blood cells and leukocytes, platelets express so-called human platelet antigens, which are identified by studying platelet alloantibodies in cases of fetal neonatal alloimmune thrombocytopenia (FNAIT) or platelet refractoriness. In this chapter, we present an overview on HPA nomenclature, expression, and distribution worldwide and where to find useful information.

6.2 Human Platelet Antigens

For decades, platelet antigens were named according to the family name, related to the individual that produced alloantibodies against certain antigens. The International Platelet Antigen Working Party introduced the HPA system in 1990. The nomenclature was revised in 2003 [1]. In the Human Platelet Antigen (HPA) nomenclature system, antigens identified on structures expressed by platelets are

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chronologically numbered HPA-1, -2, -3, etc. During the years, antigens are identified and described and may be added to the list if also the molecular basis is resolved. The Human Platelet Antigen Database can be accessed online [2] (http://www.versiti.org/HPA) and presents an overview on all named HPA, including the original name, the glycoprotein and amino acid variation underlying the antigen, the CD name of the structure carrying the antigen, and the publications first describing the antigen. Here, one can also find the information how to propose a new HPA. In this procedure, one can send in a form with the structure and responsible amino acid change underlying the antigen; the CD number of the structure; and the gene, location and dbDNPr#. Furthermore, it is important that two reference laboratories have confirmed the finding and their test results should also be made available. In this way, mistakes are optimally prevented.

The changes underlying HPAs are most often single amino acid changes, caused by single nucleotide variation (SNV) at the genomic level. Per definition, since an antigen is defined as a structure recognized by antibodies, all HPAs have led to the formation of alloantibodies by one or more individuals. For all involved genes, there is co-dominant expression. The HPAs are categorized into a total of six biallelic groups: HPA-1, HPA-2, HPA-3, HPA-4, HPA-5, and HPA-15. Moreover, classification has been made based on its occurrence in the population. The antigen with the higher frequency is labelled with "a" (e.g. HPA-1a), and the HPA with a lower frequency is labelled with "b" (e.g. HPA-1b). A "w" designation is added after the antigen name if no alloantibody against the antithetical antigen has been reported [1]. Until present, 41 different HPAs have been identified (Table 6.1) [2].

The identified antigens are located on various type of structures: the glycoprotein (GP) IIb/IIIa: αIIbβ3, CD41/CD61 or the fibrinogen receptor; GPIb/IX/V: CD42c/CD42a/CD42d or von Willebrand receptor, GPIa/IIa: α2β1, CD49b/CD29 or Very Late Antigen-2; binds to collagen/laminin and CD109, which is an important regulator of TGF-beta receptor expression. Figures 6.1a–c illustrate the HPAs expressed by these three structures. Although HPA was identified by studying platelets, the structures carrying those antigens are also expressed by other cell types. For example, the β3-chain (GPIIIa, CD61) is expressed in complex with αIIb (GPIIb, CD41) by platelets, but also with αV (CD52) by platelets, endothelial cells, and, for example, the syncytiotrophoblast of the placenta. The GPIa/IIa complex can also be expressed on activated T-lymphocytes and endothelial cells.

The proportion of individuals expressing a particular HPA varies according to their ancestry. Some of these differences in frequencies of HPA alloantigens in different populations are shown in Table 6.2. This clarifies why some platelet HPA alloantibody-related diseases are more occurring in one population than others.

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Table 6.1 Human platelet antigens and carrier glycoprotein.

Antithetical antigen	Antigen	Glycoprotein	CD
HPA-1	HPA-1a	GPIIIa	CD61
	HPA-1b		
HPA-2	HPA-2a	GPIb	CD42b
	HPA-2b		
HPA-3	HPA-3a	GPIIb	CD41
	HPA-3b		
HPA-4	HPA-4a	GPIIIa	CD61
	HPA-4b		
HPA-5	HPA-5a	GPIa	CD49b
	HPA-5b		
HPA-15	HPA-15a	CD109	CD109
	HPA-15b		
No antithetical antigen			
	HPA-6bw	GPIIIa	CD61
	HPA-7bw	GPIIIa	CD61
	HPA-8bw	GPIIIa	CD61
	HPA-9bw	GPIIb	CD41
	HPA-10bw	GPIIIa	CD61
	HPA-11bw	GPIIIa	CD61
	HPA-12bw	GPIbβ	CD42c
	HPA-13bw	GPIIIa	CD49b
	HPA-14b	GPIIIa	CD61
	HPA-16b	GPIIIa	CD61
	HPA-17b	GPIIIa	CD61
	HPA-18b	GPIa	CD49b
	HPA-19b	GPIIIa	CD61
	HPA-20b	GPIIb	CD41
	HPA-21b	GPIIIa	CD61
	HPA-22b	GPIIb	CD41
	HPA-23b	GPIIIa	CD61
	HPA-24b	GPIIb	CD41
	HPA-25b	GPIa	CD49b
	HPA-26b	GPIIIa	CD61
	HPA-27b	GPIIb	CD41
	HPA-28b	GPIIb	CD41
	HPA-29b	GPIIIa	CD61
	HPA-30b	GPIIb	CD41
	HPA-31b	GPIX	CD42a
	HPA-32b	GPIIIa	CD61
	HPA-33b	GPIIIa	CD61
	HPA-34b	GPIIIa	CD61
	HPA-35b	GPIIIa	CD61

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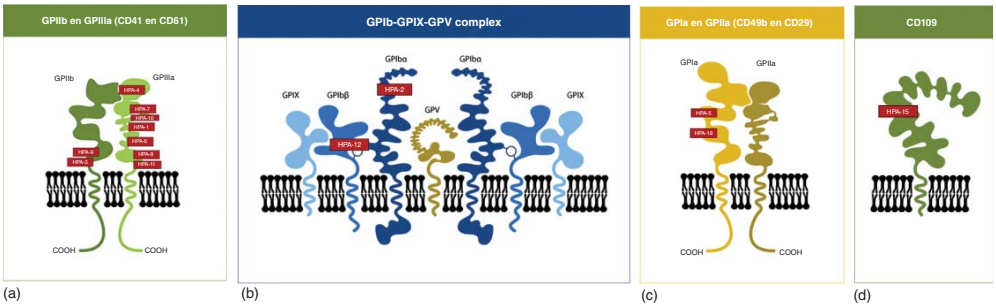

Figure 6.1 (a) GPIIb/IIIa carrying the HPA1 and HPA3 system and many HPAs, (b) GPIb/IX/V carrying the HPA-2 system and some other HPA, (c) GPIa/IIa carrying the HPA-5 system and some other HPA, (d) CD109 carrying the HPA-15 system.

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Table 6.2 Human platelet antigens and frequency distribution in people from White, Black and Asian ancestry.

System	Phenotype	White ancestry (%)	Black ancestry (%)	Asian ancestry (%)
HPA-1	HPA-1aa	72	83	99
	HPA-1bb	2	2	<<<
	HPA-1ab	26	15	1
HPA-2	HPA-2aa	85	65	90
	HPA-2bb	1	4	<<<
	HPA-2ab	14	31	10
HPA-3	HPA-3aa	37	45	36
	HPA-3bb	16	12	17
	HPA-3ab	47	43	47
HPA-4	HPA-4aa	>99.9	100	99
	HPA-4bb	<<<	0	<<<
	HPA-4ab	<<<	0	1
HPA-5	HPA-5aa	85	76	97
	HPA-5bb	1	1	<<<
	HPA-5ab	14	23	3
HPA-15	HPA-15aa	35	—	28
	HPA-15bb	23	—	12
	HPA-15ab	42	—	50

Source: Adapted from http://www.versiti.org/HPA [2].

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7

Other Polymorphic Systems

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7.1 Introduction

The recognition of pathogens in the human body and the immune mechanisms for combating them have been clarified with the discovery of the classical Human Leukocyte Antigen (HLA) molecules of classes I and II. The genes encoding these antigens are located on the short arm of chromosome 6 and exhibit significant allelic diversity, allowing a wide range of autologous (self) or allogeneic (nonself or foreign) peptides to be presented on the cell surface and recognized by immune effector cells. As a result, HLA molecules are the key structural components for triggering an immune response. Intracellular (endogenous) antigens, typically degraded by the proteasome into eight to ten amino acids, are presented by HLA Class I (HLA-Ia). In contrast, extracellular (exogenous) antigens, processed in lysosomes, are presented via HLA class II. The recognition of alloantigens and T cell activation occur via the T cell receptor, with the CD8 co-receptor binding to HLA-Ia, or the CD4 co-receptor binding to the HLA class II molecule. According to the "missing self" theory, cells that lack HLA-I on their cell surface can be recognized and specifically destroyed by natural killer (NK) cells as the absence of HLA-I indicates abnormal or stressed cells. In addition, NK cells can also recognize HLA-like molecules functioning as stress signals such as MHC class I chain-related proteins A and B (MICA and MICB).

7.2 The Structure of HLA-like MICA and MICB Molecules

HLA-like genes, such as MICA and MICB, are located between the HLA class II and HLA class I regions on the short arm of chromosome 6. In contrast, five MIC pseudogenes (MICC, MICD, MICE, MICF, and MICG) are situated in the HLA class I region.

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The MICA and MICB genes consist of 5 introns and 6 exons, with exon 1 encoding the leader peptide and the remaining exons coding for a polypeptide with about 386 amino acids. Both MICA and MICB molecules are structurally similar to HLA-Ia molecules, with three extracellular alpha domains, a transmembrane domain, and a cytoplasmic domain, encoded by exons 2–4, exon 5, and exon 6, respectively. In addition, MICA and MICB share an amino acid homology of 18–30% to HLA-Ia in their extracellular part.

7.3 The Functions of HLA-like MICA and MICB Molecules

Regarding functionality [1, 2], MICA and MICB (MICA/B) molecules do not associate with β2-microglobulin (β2m); as a result, they cannot form peptide-binding clefts. Therefore, MICA/B cannot bind or present peptides on the cell surface. Consequently, their surface expression is independent of any transporter associated with antigen processing (TAP). Since MICA/B do not present peptides, these molecules are unable to interact directly with the T cell receptor (TCR) and cannot trigger a T cell-mediated immune response. Instead, the activating C-type lectin-like natural killer (NK) group 2 member D (NKG2D) receptor recognizes specifically MICA/B in combination with a transmembrane signaling adaptor protein (DAP10) [2, 3]. This receptor is expressed as a homodimer on all NK cells, most NKT, the majority of $CD8^+$ αβT cells and γδT cells as well as on a small subset of CD4 αβ T cells with regulatory functions [4]. Thus, the MIC/B molecules together with NKG2D represent an important ligand–receptor axis that connects innate and adaptive immunity. NKG2D is a key receptor for the induction of effector immune response of NK cells in the periphery. In addition, the engagement with MICA/B can lower the activation threshold for several other NK cell receptors [5]. At variance to NK cells, NKG2D on γδT cells and $CD8^+$ αβT cells can only provide co-stimulatory signals that enhance T cell receptor responsiveness, increasing the production of IFN-γ, TNF-α, IL-2, or chemokine as well as an enhanced cytolytic response of T cells [6, 7]. On the other hand, persistent chronic exposure to MICA/B or low expression levels can impair responsiveness of several other receptors in NK and T cells. Thus, the MIC/B-NKG2D ligand–receptor axis seems to be a master regulator of immune activation thresholds, thus contributing to peripheral tolerance.

7.4 The Expression of HLA-like MICA and MICB Molecules

Despite this structural similarity to HLA-Ia molecules, MICA and MICB differ significantly in their tissue distribution [8–10]. While HLA-Ia is ubiquitously expressed on all nucleated cells outside of immune-privileged regions, MICA/B molecules show a restricted tissue distribution. They are not found on peripheral lymphocytes, but expressed on endothelial cells, epithelial cells in the gastrointestinal tract, fibroblasts, monocytes, keratinocytes, and dendritic cells. Here, MICA/B

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surface expression can be upregulated in response to stress, cell damage, infection, or malignant transformation via various posttranscriptional or posttranslational mechanisms. Their expression on tumor cells justifies that MICA/B can be considered as tumor-associated antigens. In addition to their membrane-bound forms, MICA/B can also exist in soluble forms, either as shed molecules or as part of exosome-like vesicles circulating in the peripheral blood [11, 12]. The release of soluble (s)MICA/B is mediated by proteolytic shedding via various metalloprotease including ADAM10, ADAM17, and MMP14 [13–15] or by exosome secretion [16]. The reduced expression of MICA/B on the cell surface after shedding, along with the interaction of sMICA/B molecules with NKG2D, which promotes its downregulation or blockade, impairs NK cell effector functions and facilitates immune escape. These mechanisms are discussed to be operative in solid organ and hematopoietic stem cell transplantation, autoimmunity, and malignancy.

7.5 The Polymorphisms of HLA-like MICA and MICB Molecules and Functional Consequence

MICA/B exhibits a limited number of allele or protein variations compared to HLA-Ia (HLA-A, -B, C). The MICA/B genes are located centromeric to the HLA-B [17], leading to a strong linkage disequilibrium between MICA/B and HLA-B alleles. For MICA, 570 different alleles, 9 null alleles, and 281 unique different proteins are known, while for MICB 295 alleles, 25 null alleles and 49 proteins have been described (July 2024, HLA Nomenclature@hla.alleles.org). The amino acid variations in MICA/B occurs across all three α-domains, whereas in HLA-Ia molecules, the majority of polymorphic residues cluster around the peptide binding cleft and TCR interfaces in the α1 and α2 domains. The α3 domain, which represents the docking region of the CD8/CD4 co-receptor in HLA-Ia molecules, tends to have a conserved amino acid sequence with only a few amino acid substitutions.

The polymorphic nature of MICA/B influences (i) its interaction potential with the NKG2D receptor, (ii) its expression on the cell surface, and (iii) its release in soluble form. Of all the polymorphic residues, the amino acid substitution of valine (Val) to methionine (Met) at position 129 in the α2 domain of MICA is functionally the most important. This substitution alters the binding affinity to the NKG2D receptor, resulting in MICA being clustered into forms with high (MICA-129 Met) and low binding properties (MICA-129 Val). Consequently, NKG2D-mediated intracellular signaling is enhanced by MICA-129 Met compared to MICA-129 Val, leading to increased NK cell cytotoxicity, cytokine production, and a stronger antigen-dependent co-stimulation of CD8 T cells. However, due to intracellular retention, MICA-129 Met is expressed at lower levels on the cell surface and is more susceptible to proteolytic membrane shedding [18]. In case of high expression intensities of MICA-129 Met, this may lead to more efficient downregulation of the NKG2D receptor on NK and T cells compared to MICA-129 Val, impairing effector function in NK cells, and co-stimulatory function in T cells [19].

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In addition to the polymorphic sites in exons that code for the extracellular domains and thus define the MICA alleles, the MICA gene shows microsatellite variations in exon 5 encoding 4 up to 11 alanine repeats in the transmembrane domain. The number of alanine residues differs by the number of triplet-repeats in exon 5. One unusual microsatellite variant, designated MICA-A5.1, has five triplet repeats with an additional guanine insertion in exon 5, causing a frameshift mutation and a premature stop codon in the transmembrane region. This results in a truncated MICA protein that lacks 10 amino acids in the transmembrane region and the cytoplasmic domain [20]. MICA alleles harboring the MICA-A5.1 variant such as the most common MICA*008 allele seem to exhibit high cell surface expression, causing an increased NKG2D receptor interaction [21]. Interestingly, MICA molecules having the A5.1 variant are delivered from cells via exosomes [16].

Further polymorphisms have been described in the 5′untranslated region (UTR) of the proximal promoter region [22] as well as in the 3′UTR region for MICA/B [23], which increases the complexity of transcriptional and posttranscriptional regulation of MICA/B expression under physiological and pathological conditions.

Nevertheless, MICA/B molecules, whose expression is controlled by polymorphisms in the 5′UTR, 3′UTR, and exons 2–5, can represent targets for alloimmune responses, particularly in the setting of allogeneic transplantation. In solid organ transplantation, a mismatch in MICA/B between the recipient and donor can trigger or intensify both cellular and humoral allograft rejection. In addition, proven pre-sensitization of kidney transplant recipients to MICA antigens is significantly associated with a reduced allograft survival (see Chapter 25) following transplantation [24].

7.6 Nonclassical HLA Class I

In addition to the HLA-like MIC genes, there exists another district subset of genes in the polymorphic HLA class I region that encode for so-called nonclassical HLA molecules, known as HLA-Ib. This group includes HLA-E, HLA-G, and HLA-F. The HLA-E gene is positioned between HLA-C and HLA-A, while the genes for HLA-G and -F are located telomeric to HLA-A.

7.7 Key Differences Between Nonclassical HLA Class I and Classical HLA Class I Molecules

All members of the HLA-Ib family differ from classical HLA class I molecules in several key aspects. They exhibit significantly lower allelic variations compared to HLA-Ia. In addition, the expression of the various HLA-Ib molecules differs in terms of tissue distribution and intensity, resulting in different expression profiles compared to HLA-Ia. The differential tissue expression of the HLA-Ib molecules is due to nucleotide sequence variations in the regulatory elements of the HLA-E, HLA-F, or HLA-G promoters. These variations lead to differential reactivity

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toward nuclear factor "kappa-light-chain-enhancer" of activated B-cells (NFκB) or interferon regulatory factor1 (IRF1), and class II transactivator (CIITA)-mediated induction [25].

Despite these differences, the protein structure of HLA-Ib is very similar to that of HLA-Ia. The three alpha domains of the heavy chain of HLA-E, -G, or -F can form a peptide binding cleft, which can be stabilized by ß2m, thus in principle enabling peptide loading. However, due to a limited number of amino acid exchanges within the α1 and α2 domains, HLA-Ib molecules can present only a limited peptide repertoire on the cell surface. Consequently, while the recognition of allogeneic peptides and the activation of alloimmune responses via the TCR are possible, they are limited to a small number of alloantigens, if any. Therefore, HLA-Ib molecules must serve additional functions compared to HLA-Ia in both the innate and adaptive immune systems.

7.8 The Expression of HLA-E Molecules

Overall, HLA-E is co-expressed with HLA-Ia on most nucleated cells, but with a significantly lower expression intensity than HLA-Ia [26]. The highest levels of expression are found at immune-privileged sites, such as placental trophoblasts, where HLA-Ia molecules are not expressed under physiological conditions, as well as in leukocytes and endothelial cells. In addition, HLA-E is released from cells by metalloproteinase-mediated shedding or secretion via extracellular vesicles. Soluble forms of HLA-E (sHLA-E) are present in peripheral blood and other body fluids and are discussed to be involved in immune regulation [27–29].

Under conditions of stress, in malignant transformation, infection, and allo-transplantation, HLA-E expression is often upregulated. This upregulation is partly caused by gene transcription mechanisms triggered by pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), or interferon-γ (IFN-γ), which induce both the upregulation of HLA-E surface expression and the release of soluble HLA-E molecules. The upstream STAT1 binding site enables the induction of HLA-E expression by IFN-γ [30]. However, in certain cancers, HLA-E is constitutively upregulated independent of IFN-γ. Additionally, HLA-E transcription can also be induced by CIITA via the SXY regulatory module, while HLA-E is not inducible by NFκB or IRF1 as is HLA-Ia [25].

7.9 The HLA-E Peptide Presentation Controls the Functional Interaction with NK and T Cells

Importantly, HLA-E can only form a rigid, inflexible binding pocket, which allows only a few peptides to bind, leading to a restricted variety of peptides displayed on the cell surface. Under physiological conditions, HLA-E presents peptides derived from the signal leader sequences of HLA-A, -B, -C, and -G. Thus, the HLA-E expression level is directly linked to the availability, i.e. the expression

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levels, of HLA-Ia or HLA-G peptides. However, the binding affinities of signal leader peptides from different HLA-Ia alleles are not uniform. Consequently, stable HLA-E expression depends on the corresponding HLA-Ia alleles with the respective leader sequences [31].

HLA-E primarily acts as a ligand for the inhibitory NKG2A or activating NKG2C receptor, which are generally expressed on NK cells, NKT cells, and as specific subset of CD8+T cells. Both receptors are expressed on the cell surface as heterodimers with the non-signaling CD94 molecule [32]. NKG2A possesses immunoreceptor tyrosine-based inhibition motif (ITIM) elements in its cytoplasmic tail, enabling the downstream transmission of inhibitory signals. In contrast, NKG2C non-covalently associates with the membrane receptor DAP12, which carries an activating ITAM motif in the transmembrane region [33]. CD94 is required for the transport of NKG2A or NKG2C to the cell surface [34] and for the recognition of peptides presented by HLA-E. In most cases, the HLA-E/peptide complex binds with a six-fold higher affinity to the CD94-NKG2A heterodimer than to the CD94-NKG2C [35]. However, the extent of CD94-NKG2A/C receptor recognition is determined by the peptides loaded onto HLA-E [31].

Since the signal leader peptides of HLA-Ia and HLA-G form the main peptide pool for HLA-E in healthy cells, the HLA-E/peptide complex reflects HLA class I biosynthesis and expression. In this sense, NK cells remain tolerant to autologous cells due to the interaction of HLA-E/peptide with CD94-NKG2A. Thus, HLA-E/peptide together with CD94-NKG2A represents an important immune checkpoint axis in balancing NK cell activity. However, intracellular pathogens like human cytomegalovirus (HCMV) [36, 37] and other viruses [38], as well as malignant cells, often escape immune surveillance by cytotoxic T cells through downregulation HLA-Ia, resulting in alteration or reduction of the HLA-Ia-derived peptide pools. As a consequence, the HLA-E/peptide-mediated inhibitory signaling pathway via CD94-NKG2A may be abolished, allowing for the activation of NK cells via various activating receptors, according to the missing self hypothesis. To circumvent such NK cell-mediated immune responses, malignant or infected cells utilize mechanisms that allow exclusive HLA-E expression [39] on the cell surface, such as providing peptides that mimic functional HLA-Ia-peptides for loading onto HLA-E. Even if the antigen processing machinery of HLA-Ia, e.g. the TAP function, is impaired, certain peptides with canonical (8–10 amino acids) or non-canonical properties (up to 16 amino acids in length) can be bound and stable presented by HLA-E [40]. Thus, in contrast to HLA-Ia, stable HLA-E surface expression and peptide presentation can occur independently of proper functionality of the antigen processing machinery.

In the context of HCMV infection, the UL-40-derived peptide presented by HLA-E can also stimulate cytotoxicity and cytokine production of NK cells after interaction with the activating NKG2C receptor heterodimer, thereby controlling HCMV spread. NK cells that have responded to HCMV infection via this pathway are able to develop a specific clonal expansion and a secondary memory response to HCMV reinfection [41]. Such NK cells are called adaptive NK cells.

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Thus, HLA-E/viral-derived peptide complex together with CD94-NKG2C heterodimer represents an important ligand–receptor axis in linking the innate and adaptive immunity.

Under pathological conditions, the availability of alloantigens in the peptide pool of a cell increases, leading to enhanced presentation of allo-peptides by HLA-E. In this case, the direct interaction of HLA-E with TCR can trigger T-cell-mediated immune response that can lead to cytotoxic elimination of HLA-E-presenting target cells and clonal expansion of alloreactive CD8 T cells [42]. Thus, the formation of HLA-E/allo-peptide complexes has the potential to function like HLA-Ia as an antigenic determinant for the TCR in the adaptive immune system.

7.10 The Polymorphisms of HLA-E and Its Influence on Gene Expression

Among HLA-Ib, HLA-E has the greatest biological diversity, with 366 alleles, 140 different proteins, and 10 non-expressed molecules (July 2024, HLA Nomenclaturehla .alleles.org). However, only two alleles, *HLA-E*01:01* and *HLA-E*01:03*, dominate in different ethnic populations. The frequency of each is over 48% [43, 44]. These two HLA-E alleles vary only by a single amino acid substitution of arginine for glycine at position 107 (HLA-E107R and HLA E107G) within the α2 domain of the heavy chain [26]. This exchange does not fundamentally alter the peptide binding cleft of the corresponding proteins but affects the thermal stability of HLA-E and thus the cell surface expression. Indeed, the *HLA-E*01:03* is expressed with a higher intensity than the *HLA-E*01:01*. Consequently, such differences in the expression level of the two alleles affect the strength of receptor recognition (CD94-NKG2A/C or TCR) on NK or T cells. In fact, these alleles are associated with a number of diseases, including viral and bacterial infections, cancer, and autoimmune disorders [45–48].

There are other polymorphisms in the coding or noncoding HLA-E gene regions that can influence its expression. In particular, the 5′ upstream segment and the 3′UTR of HLA-E show high variability, which may indirectly impact protein expression by affecting processes like DNA transcription [49–51]. In the context of the functional role of HLA-E, differences in protein expression, such as those between HLA-E*01:01 and HLA-E*01:03, may significantly affect the immune system's ability to react. Variation in these regions has been reported to be associated with the occurrence and outcome of inflammatory diseases, leukemia, and viral infections [51–55].

7.11 The Expression of HLA-G

The gene for HLA-G, which consists of eight exons and seven introns, was first described in 1987 [56], and in 1990, its expression was detected on semi-allogeneic extravillous trophoblast cells (cytotrophoblasts) at the maternal-fetal interface

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[57]. These cells preferentially express HLA-G, and to a lesser extent HLA-E and HLA-F, but do not express the classical HLA-A and B, nor HLA class II (HLA-DR, -DQ, -DP). This unusual expression pattern led to the concept that HLA-G is a key molecule in embryo implantation by locally shaping the immune system into tolerance during an allogeneic situation such as pregnancy, where the mother's immune system must accept the fetus. Later studies found high expression density of HLA-G in other immune-privileged regions of the body such as the thymus, cornea, nail matrix, erythroblasts during hematopoiesis and pancreas. Additionally, HLA-G-specific transcripts were found in almost all cell types, including the fetal liver, myelomonocytic cells, fetal and adult eye tissue, skin, and keratinocytes as well as peripheral blood lymphocytes. Soluble HLA-G molecules (sHLA-G) are found in almost all body fluids, especially in peripheral blood [58, 59]. Certain conditions, such as cell activation or disease, can cause HLA-G or sHLA-G to be newly expressed or abnormally produced. As a result, HLA-G/sHLA-G has been linked to the development of cancer, infections, inflammatory diseases, autoimmune disorders, and complications during pregnancy. On the other hand, high HLA-G/sHLA-G expression in the allograft favors the graft acceptance after transplantation.

7.12 The Unique Structural Features of HLA-G

The exon/intron structure of HLA-G is similar to classical HLA-Ia genes, but with important differences. For instance, exon 6 contains a premature stop codon and an extended 3′ untranslated region (UTR) largely consisting of exon 8. Due to the premature stop codon the cytoplasmic part is reduced to six amino acids. This truncation leads to the loss of specific signaling sequences for endocytosis, which prolongs the half-life of HLA-G on the cell surface. In addition, the shortening creates a so-called di-lysine motif, which is responsible for ensuring that only HLA-G molecules with high-affinity peptides reach the cell surface [60].

HLA-G can exist in different molecular forms due to alternative splicing. Besides the full-length HLA-G gene product (HLA G1), six additional HLA-G transcripts (HLA-G2, -G3, -G4, -G5, -G6, and -G7) can be generated. Each isoform differs in which domains they contain, such as HLA-G2, which has the α1 and α3 domains. HLA-G4 contains the α1 and α2 domains, while HLA-G3 consists only of the α3 domain. The secreted isoforms miss the transmembrane and cytoplasmic domain but retain either intron 4 (HLA-G5 and -G6) or intron 2 (HLA-G7). HLA-G6 and HLA-G7 represent the secreted counterpart to HLA-G2 and HLA-G3, respectively.

Due to an unpaired cysteine residue at position 42 in the α1 domain or at position 147 in the α2 domain, HLA-G isoforms having α1 or α2 domains can form dimers or oligomers through the formation of an intermolecular disulfide bond [61].

Like classical HLA class I molecules, HLA-G1 and HLA-G5 assembles with β2m and a nona-peptide in the endoplasmic reticulum (ER). Nevertheless, versions of HLA-G proteins from both the primary and spliced transcripts can also exist as βm-free HLA-G molecules.

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7.13 The Functions of HLA-G are Directed by the Structural Features

The specific receptors of HLA-G are as follows:

i) the inhibitory receptor ILT2/CD85j/LILRB1 (ILT2), which is expressed by NK cells, B and T lymphocytes, and antigen-presenting cells (APC);
ii) the inhibitory receptor ILT4/CD85d/LILRB2 (ILT4), which is expressed by monocytes, dendritic cells (DC), or other APC;
iii) the KIR2DL4/CD158d (KIR2DL4) receptor, which is preferentially expressed on decidual NK cells, suggesting a specific role in pregnancy; and
iv) the CD8 co-receptor found on subpopulation of T and NK cells [58].

HLA-G isoforms containing the α3 domain can interact with CD8, ILT2 orILT4. The association of HLA-G with ß2m controls the interaction with the ILT2/4 receptors: ß2m-associated HLA-G molecules specifically interact with ILT2, whereas β2m-free HLA-G molecules are targeted byILT4. Of importance, HLA-G dimers show a higher affinity and avidity for the ILT2/4 receptors than monomers, leading to a more efficient immunosuppressive signaling [62].

When ß2-m-associated HLA-G binds to ILT2, it initiates short-term tolerance locally by inhibiting cytotoxicity of T and NK cells, proliferation of T and B cells, B cell activation and their antibody production. In addition, HLA-G modulates the cytokine release of mononuclear cells, resulting in a shift in the Th1/Th2 equilibrium toward a relative Th2 dominance. Indirectly, HLA-G contributes to the induction of short-term tolerance by providing signal leader peptides for stable HLA-E surface expression that enable HLA-E to interact with inhibitory CD94-NKG2A receptor pairs on T and NK cell subsets. The binding of sHLA-G molecules to the CD8 co-receptor triggers Fas/FasL-mediated apoptosis in subgroups of CD8-carrying T and NK cells.

Beyond short-term tolerance, HLA-G can induce long-term tolerance by generating different types of regulatory T cells (Treg). For instance, allo-specific Tregs can be generated by the interaction of β2-free or β2-associated HLA-G1 with ILT2/4 on so-called DC-10 cells. HLA-G-bearing DC or HLA-G5 can induce suppressor T cells in the presence of IL-10 that exhibit only marginal CD4 or CD8 co-receptor expression. These types of Treg cells seem to accumulate on sides of inflammation.

HLA-G isoforms with the α1 domain are able to interact with KIR2DL4. The binding avidity toward KIR2DL4 is independent of the monomeric/dimeric status of HLA-G molecules. However, the functional outcome of such an interaction is difficult to predict in in-vivo situations due to the inhibitory element in the cytoplasmatic tail of KIR2DL4 and the activating element in its transmembrane region. It is discussed that binding to KIR2DL4 elicits the secretion of pro-inflammatory cytokines, chemokines, and angiogenic factors but leads to only marginal cytotoxicity in resting NK cells [63, 64].

Finally, the presence of HLA-G mediates the increased expression of its own receptors (ILT2, ILT4 and KIR2DL4) on APC, T and NK cells, thereby enhancing its own inhibitory potential [65]. This upregulation seems not to require any antigenic

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receptor. In addition, priming peripheral blood cells with purified soluble HLA-G1 (sHLA-G1) molecules prior to T cell activation results in an increased abundance of CD8 T cells expressing ILT2 co-expressing immune checkpoint receptors such as programmed cell death protein-1 (PD-1), cytotoxic T lymphocyte-associated protein (CTLA-4), T cell immunoglobulin, mucin domain-3 (TIM-3), or CD95. Thus, priming with sHLA-G1 induces a pronounced immunosuppressive/exhausted phenotype of CD8 T cells after T cell activation [66].

Taken together, HLA-G has the capacity to modulate the effector function of almost all immune cells of the adaptive and innate immune system through a broad spectrum of inhibitory receptors, more effectively than other checkpoint ligands. Both, the structural diversity of HLA-G and the broad spectrum of specific inhibitory receptors, allow a (local) fine-tuning of effector function in various (tissue-resident) immune cell subsets.

7.14 The Polymorphisms of HLA-G and Its Impact on Gene Expression

HLA-G is more highly conserved in the coding nucleotide sequences than HLA-E. To date, 169 alleles, 51 different proteins and 6 unexpressed molecules have been described (July 2024, HLA Nomenclaturehla.alleles.org). However, the majority of alleles are rare, and only five alleles are observed worldwide with a cumulative frequency of 98% [67]. In contrast to the coding regions, the introns and regulatory regions of the 3′-UTR and the promoter region (5′- upstream regulatory region [URR]) are highly variable.

HLA-G expression levels are controlled by posttranscriptional mechanisms, including alternative splicing and microRNAs (miRNA). These miRNAs can modify the expression by promoting either mRNA degradation or translation blockage. The most important miRNAs regulating HLA-G expression are: miR-148a, miR-148b, miR-152, miR-133a, miR-628-5p, and miR-548q [68]. Variation in the target sequence of miRNA can influence their binding strength and thereby gene expression. Thus, the 3′UTR, which is the primary target for miRNA, represents key segment for posttranscriptional regulation or controlling of HLA-G expression due to its polymorphic sites.

HLA-G 3′UTR displays 27 nucleotide variations (see chapter 25), 8 of which are single nucleotide polymorphisms (SNP), as well as the presence or absence of a 14-bp fragment (14bp INS/DEL) at the beginning of 3′UTR segment, display frequencies greater than 1%. Initially, the 14-bp insertion variant was associated with low protein levels of membrane-bound and soluble HLA-G. A number of studies have investigated the relevance of 14bp INS/DEL and the other variants in clinical trials examining pregnancy complication, autoimmune diseases, cancer, infection, transplantation related complication or therapy response. Overall, due to the heterogeneity of the studies, consistent results on clinical significance could not be demonstrated when only a single SNP was considered.

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Although studies on individual SNPs have not shown consistent clinical significance, the 27 variants can arrange to 35 distinct 3′UTR haplotypes designated as UTR-01, UTR-02 and so forth. Nine haplotypes account for more than 95% of all observed haplotypes, with UTR-1 and UTR-2 being the most common. Concerning HLA-G expression, the UTR-01 haplotype seems to be associated with higher expression of HLA-G/sHLA-G, whereas UTR-02 and UTR-05 are linked to lower levels. With respect to allogeneic situations, the UTR-2 donor haplotype has been adversely associated with an increased risk of acute cellular and antibody mediated rejection after living-donor kidney transplantation [69]. Meanwhile, UTR-01 and UTR-04 haplotypes appear to exert a protective role against recurrent miscarriage [70, 71] or antibody-mediated rejection. Conversely, UTR-04 promotes an adverse disease course in locally advanced, non-metastatic breast cancer [72] or viral infection complication such as BK polyomavirus replication or nephropathy in living-kidney transplantation [69].

The HLA-G promoter region is unique among class I HLA genes due to its interactions with specific transcription factors that are activated by extracellular stimuli such as heat shock, hypoxia, and certain hormones such as glucocorticoids and progesterone. Progesterone is particularly essential for the maintenance of endometrium and embryo implantation [73]. The promoter region includes a hypoxia-regulatory element (HRE), a heat shock element, and a unique progesterone response element (PRE). Additionally, the enhancer A (EnhA) region binds specifically to p50/p50 homodimers of NF-κB, while a deletion in the interferon-stimulated response element (ISRE) allows precise regulation of HLA-G protein levels by altering the binding affinity of transcription factors [74]. To date, 35 SNP have been described in HLA-G promoter region, which can arrange to 64 haplotypes. However, nine haplotypes account for more than 95% of all haplotypes [67]. Genetic variants in the promoter region are involved in developing of any forms of graft versus host disease (GvHD) after hematopoietic stem cell transplantation, as well as of idiopathic recurrent spontaneous abortions, and cancer [75, 76]. Studies indicate that certain extended haplotypes are associated with cancer development and morbidity as well as with the expression of the HLA-G protein [77, 78].

7.15 The Expression of HLA-F

In contrast to the other HLA molecules, HLA-F is preferentially localized intracellularly in the ER or Golgi apparatus in resting lymphocytes and monocytes. It is also present in certain tissues such as tonsils, spleen, thymus, trophoblasts and fetal liver. However, HLA-F surface expression can occur in activated T, NK and B cells, as well as in virus-infected cells and on tissues such as insulin-containing islets or during the first and second trimester of pregnancy on invasive extravillous trophoblast cells [79]. In addition to intracellular and surface-bound expression, HLA-F molecules are also detected as circulating soluble molecules in peripheral blood [80]. Like other HLA-Ib molecules, abnormal HLA-F expression is detected

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under pathological conditions such as cancer, infections, impaired pregnancy and autoimmune diseases, which is often associated with disease progression or an unfavorable disease course [79].

7.16 The Functions of HLA-F are Directed by Its Structural Features

The HLA-F gene is organized similarly to that of other HLA-Ia molecules, with the exception that exon 7 remains untranslated, resulting in a truncated cytoplasmic tail. There are also different HLA-F isoforms due to variation in the lengths of the cytoplasmic tail [81]. Interestingly, HLA-F surface expression is independent of peptide loading machinery and peptide loading. In this case, the transport to the surface is facilitated by two different motifs in the cytoplasmic tail of HLA-F, which enable the transport out of the ER and the forward transport through the cis-Golgi apparatus, respectively [80]. As a result, the HLA-F heavy chain can be expressed as an open conformer without ß2m and peptide on the surface or together with ß2m and peptide as a trimeric complex, similar to other HLA class I molecules. When loaded with peptides, HLA-F typically presents endogenous peptides originating from either the cytosol or the nucleus a preferred length of 16 and sometimes even longer residues, similar to HLA class II molecules [82]. These long peptides protrude from the peptide binding groove and thus expand the accessible surface for corresponding receptors [83].

Regarding functional consequences, HLA-F acts as an immune-regulating molecule that can bind to inhibitory as well as to activating receptors on NK and T cells, depending on its conformal structure and peptide loading. In particular, open HLA-F structures can interact with the inhibitory receptors KIR3DL2 and ILT-4 as well as with the activating receptors KIR3DS1 and KIR2DS4. In contrast, the inhibitory receptors ILT2 interact only with closed HLA-F molecules consisting of HLA-F heavy chains, ß2m and peptides [84–86]. This means that HLA-F is able to regulate immunity by changing the conformational structure, thereby enabling the interaction potential with the other T and NK cell receptors.

7.17 The Polymorphisms of HLA-F and Its Impact on Gene Expression

HLA-F is one of the most conserved loci within the nonclassical HLA-Ib gene family. In total, 94 alleles, 19 proteins, and two null alleles are reported (July 2024, HLA Nomenclaturehla.alleles.org). Although the three most common variants *HLA-F*01:01*; **01:03* and **01:04* vary by only two amino acids in exon 2 and exon 4, respectively, the peptide repertoire of these allotypes is extremely divergent. Interestingly, *HLA-F*01:03* appears to preferentially present peptides from DNA-binding proteins. Moreover, *HLA-F*01:03* is directly linked to reduced levels of hepatitis B virus DNA in chronically infected patients [87]. *HLA-F*01:01* derived

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peptides are frequently associated with proteins that interact with HIV-1, suggesting that *HLA-F*01:01* might play a role in the immune defense against HIV infection [83]. These findings suggest that an antiviral immune response may be modified depending on the expressed HLA-F allele.

Regarding regulatory segments of the HLA-F locus [88–90], 16 or 8 variable sites are described in the distal and proximal promoter, arranging into 20 haplotypes with sequences frequencies above 1% worldwide. According to this, HLA-F coding sequences can be divided into two main HLA-F lineages. *HLA-F*01:01:01* and *01:03* allele groups have similar promoter sequences, whereas the *HLA-F*01:01:02* allele presents a divergent sequence within the promoter segment and many introns as compared to the *HLA-F*01:01:01* and *HLA-F*01:03*. Interestingly, *HLA-F*01:01:02* allele is associated with a higher HLA-F mRNA expression level. Besides the HLA-F 5′URR, the 3′UTR of HLA-F has two haplotypes highly conserved suggesting preserved function in immune regulation.

7.18 Summary

7.18.1 HLA-like Genes

MICA and MICB Molecules

- **Expression:** MICA and MICB are stress-induced molecules predominantly found on the surface of infected or abnormal cells. Their expression is upregulated in response to cellular stress signals, making them important markers for the immune system to recognize compromised cells.
- **Function in immune regulation**: Both MICA and MICB interact with the NKG2D receptor on natural killer (NK) cells and T cells. This interaction activates these immune cells, enhancing their ability to eliminate infected or transformed cells. By doing so, MICA and MICB play critical roles in immune surveillance and response. However, persistent chronic exposure to MICA/B and the presence of soluble MICA/B molecules counteract the NKG2D-mediated immune acivation.
- **Polymorphism**: MICA and MICB exhibit significant polymorphism, which enhances the immune system's adaptability to various pathogens. The variability in their genetic makeup allows for diverse immune responses, making them important in recognizing a wide range of infections and abnormalities.
- **Disease:** Altered expression of MICA and MICB is associated with various diseases, including cancer and chronic infections. Tumors can down-regulate MICA and MICB to evade detection by the immune system, highlighting their significance in immune evasion mechanisms. Additionally, changes in their levels can impact autoimmune responses, contributing to disease progression.

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7.18.2 Nonclassical HLA-Ib Molecules

HLA-E

- **Expression:** HLA-E is mainly found on the surface of immune cells like natural killer (NK) cells and T cells, as well as in the endoplasmic reticulum of resting lymphocytes. Its expression is influenced by classical HLA molecules.
- **Function in immune regulation**: HLA-E helps regulate the immune response by presenting peptides to CD94/NKG2A receptors on NK and T cells, inhibiting their activation to maintain self-tolerance. However, HLA-E loaded with viral-derived peptides can also trigger an adaptive NK cell immune response via the CD94-NKG2C receptor or a TCR-mediated T cell response.
- **Polymorphism**: HLA-E has limited polymorphism, with fewer known alleles compared to classical HLA molecules, reflecting its specialized immune function.
- **Disease:** Altered HLA-E expression can contribute to various diseases, including cancers and viral infections, by allowing tumors to evade immune detection.

HLA-G

- **Expression:** HLA-G existing in both membrane-bound and soluble forms is primarily expressed in the placenta and other immune-privileged regions under healthy condition. HLA-G neo-expression occurs in malignant transformed cells or during infection disease.
- **Function in immune regulation**: HLA-G suppresses T cell and NK cell activation by binding to specific inhibitory receptors such as ILT2, ILT4, or KIR2DL4 crucial for the inducion and maintaining of short-term or long-term immune tolerance during pregnancy and allograft acceptance after transplantation.
- **Polymorphism**: Compared to classical HLA molecules, HLA-G has limited variability in the coding region but are highly variable in regulatory regions of the 3′-UTR and the promoter region that control HLA-G expression level, which is required for the specialized role in immune regulation.
- **Disease:** Altered HLA-G expression is linked to pregnancy complications, cancers, and autoimmune disorders, helping tumors evade immune responses.

HLA-F

- **Expression:** HLA-F is primarily located within the endoplasmic reticulum and Golgi apparatus but can be expressed on activated immune cells. It exists in two forms: an open conformer and a peptide-bound form.

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- **Function in immune regulation**: HLA-F interacts with inhibitory (KIR3DL2, ILT2, ILT4) as well as activating receptors (KIR3DS1,KIR2DS4) on immune cells, modulating immune responses based on its conformation and the peptides it presents.
- **Polymorphism**: HLA-F exhibits low polymorphism and limited variability in the regulatory regions, suggesting a conserved function in immune regulation.
- **Disease:** Changes in HLA-F expression or function may be involved in autoimmune diseases, infections and transplant rejection, highlighting its potential role in therapeutic strategies.

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Part II

Clinical Applications

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8

Basic Concepts of Organ Transplantation

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8.1 Introduction

This chapter discusses the inclusion of patients on a suitable waiting list, procurement of postmortem organ donors, and allocation of organs after death. The part of living donation is described when it directly affects the patient on a waiting list. The distribution of postmortem organs requires, first and foremost, their availability. The extraction, or harvesting, of organs from donors is stringently regulated, complying with the directives established by national authorities and international organizations, in addition to national laws governing the process. Every nation subscribes to its distinct religious convictions and cultural standards. Accepting or refusing a contribution is a personal decision, influenced by individual views, ideologies, and the context at the time of the request. The authors assert that there is no unequivocal correct or bad approach to decision-making. We have thus far refrained from referencing this in the report below.

The kidneys are the most frequently available organs for postmortem donation. Thoracic organs (heart and lungs), liver, pancreas, small intestine, and corneas are prioritised, with sporadic occurrences of knees, hands, faces, and other body parts. The latter will be excluded from this chapter. Interested parties are advised to reach out to the appropriate allocation or donation organizations for further information.

The authors primarily investigated relevant sources for information. It is unequivocally infeasible to cite all relevant publications or to encompass every potential viewpoint on allocation and donation. It is evidently impractical to list all potential organizations worldwide. The authors possess a personal viewpoint regarding the distribution, especially in relation to the future. This will be presented at the conclusion of the chapter and will be duly cited.

The authors assert the absence of any conflict of interest.

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8.2 Overview of the Decision-Making Procedure

Prior to any organ offer, a decision-making procedure is necessary. This primarily individual decision is depicted in Figure 8.1.

A summarizing flowchart of the complete procedure, from waiting list to transplantation is depicted in Figure 8.2.

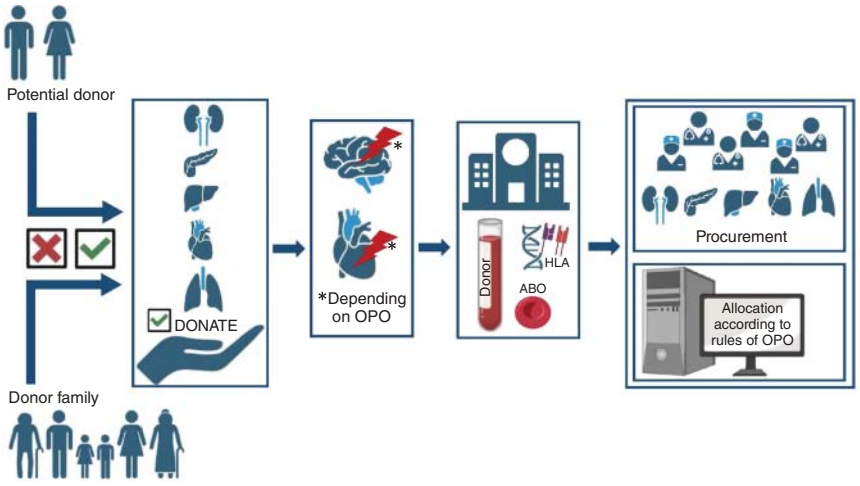

Figure 8.1 Flowchart illustrating the process from decision-making to allocation, OPO -Organ procurement organization. Source: Created with BioRender.com.

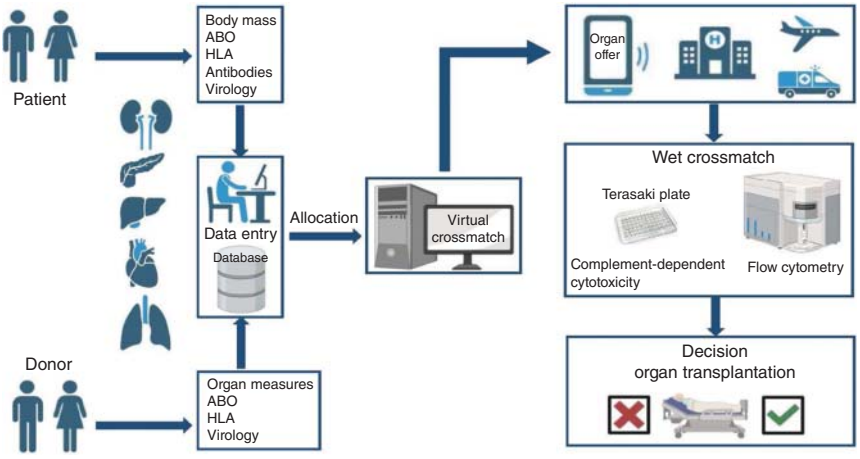

Figure 8.2 Flowchart presents a succinct graphic representation delineating the involved phases. Local and national guidelines may be applicable and can modify the program. Moreover, a wet crossmatch is superfluous for specific organs, including the liver or heart, in the case of unsensitized patients. Source: Created with BioRender.com.

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8.3 Registration and Waiting List

8.3.1 Waiting List

Patients diagnosed with end-stage organ disease, such as kidney, heart, or liver failure, are frequently given the chance to be added to an organ transplantation waiting list for post-mortem donation. When a postmortem donor is identified and an organ is assessed as appropriate for the recipient, an offer may be done. Transplantation may proceed contingent upon the patient's consent. This transformative action can only be performed through the donation of postmortem organs, either by the deceased before death or by the donor's family. Living donations may be conducted for kidneys and, in specific instances, for the liver. Various organ procurement organizations and national entities formulated recommendations. Nevertheless, numerous challenges must be surmounted.

8.3.2 Organ Transplantation

As previously stated, living donors are viable for patients in need of a kidney or liver. The kidney is the principal organ that patients in some countries can get due to socio-legal or cultural views that allow such transplants.

There are several differences between living and postmortem kidneys. The main emphasis is on the elective surgery. The organ donor and recipient are prepared and supervised, and the procedure is usually performed during daytime hours. The cold ischemia time, if any is very short. This differs from the conditions associated with post-mortem donation. The surgical surgery generally takes place at night, and renal function is primarily impaired due to the cytokine storm at the time of brain death, the conditions surrounding the death, and the extended period of cold ischemia. These characteristics do not diminish the prospective advantages of HLA matching [1, 2].

In many countries, patients are typically placed on a waiting list for the desired organ before receiving any offers. Even if the transplanted organ derives from a living donor. The fundamental idea of this phase is that, if a post-mortal donor becomes available while awaiting living transplantation, it will be employed. The ethical consideration in this case is that the living donor will not experience any damage due to the procurement procedure, i.e., narcosis, operation. The term "living donor" specifically pertains to immediate relatives. This term was widening with time and many countries worldwide accept donations also from no direct first-degree relatives, but even friends. However, every case must be proposed to the ethical committee of the institution, and upon their decision, a transplantation might occur. Currently, in several countries, including the Netherlands, the USA, Canada, and the Czech/Austrian/Israel cooperation, there are notable developments in organ donation programs. Here, the kidney is transplanted to a patient not belonging to the immediate family [3]. Additionally, cross-over or chain systems

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are utilized to aid patients in need [4]. As stated above, the living donor need not be a close relative; they may be a friend or even an acquaintance participating in an exchange program of pair donation or a chain.

A major concern with living donation is blood group incompatibility and/or a positive crossmatch before transplantation, due to donor-specific HLA antibodies. A cross-over may be utilized in this context. The donor from couple A will offer the kidney to the patient of pair B, while the donor from couple B will aid the patient of couple A. The transplantation occurs in parallel in two operation rooms of the same hospital, creating a logistic problem. Furthermore, a chain was established in the United States due to this situation [5]. A live donation occurs from one couple to their patient, then followed by the donor from this couple donating to the patient of a third couple, and so on. The chain's length may fluctuate based on the array of pairs. Both types of help are viable and predominantly regulated by the authorities. As seen earlier for the standard waiting list, primarily, patients with blood group O and those who are highly sensitized accumulate. Ongoing initiatives are necessary to support these patients. Important for the regions without option for pair donations or chains to first establish the legal requirements for this type of donation and then proceed.

8.3.3 Postmortem Contribution

When a postmortem organ donor who satisfies the criteria set by the relevant national authorities and the Organ Procurement Organization (OPO) becomes available, blood group typing is performed simultaneously with the necessary virological tests and the definition of the HLA factors. Currently, most countries and their OPO solicit HLA-A, B, C, DRB1, DRB345, DQA1, DQB1, DPA1, and DPB1. Typically, the typing is performed at a so-called low-resolution level. In specific regions, like Australia, high resolution is simultaneously attained [6]. A multicentral international study recognized the feasibility of nanopore sequencing on a large scale for HLA typing [7].

8.3.4 Distribution of the Organs

The result of the typing is entered a specific allocation algorithm together with the additional required information deemed important. All factors are compared between the organ donor and the patient. At the end, a list of suitable patients is prepared. The offer will be done following the list. In case the first patient on the list is not transplantable, which occurs in significant numbers, the immediate next patient on the list receives the offer, and so on. To ensure the opportunity that difficult patients will be offered an organ-specific programs have been established worldwide. These groups of patients are mainly pediatric, elderly patients, highly sensitized patients, and others. However, some patients with an odd HLA type to the donor population, the expression of HLA factors is usually not considered for receiving an offer. These patients might be helped via a special program. The decision is dependent on the directives of the national authorities.

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In nearly all countries, immediate relatives of the potential or selected donor are solicited for the donation, also referred to as a "gift of life." Recently, the individual can make this option alone. Brain death is a condition for any postmortem donation, if circulation remains intact. In certain cases, organs may be approved for donation following a brief cessation of circulation. This is typically referred to as "non-heart beating" or donation after cardiac death (DCD) bids, which are approved by some countries.

8.4 Procurement of the Organs

8.4.1 Ownership of the Organs

Religion significantly influences the critical issue of organ ownership. From a Christian perspective, Christians are interconnected and so have a duty to donate their organs to others when they are no longer needed [8]. The author proceeds by establishing analogies with Christ's sacrifice. A perspective on the views of the Muslim community is articulated by Ali [9]: "Organ transplantation, an accepted treatment modality that alleviates patients suffering from end-stage organ failure, remains an unresolved issue." Some Muslim academics and jurists advocate for organ donation and transplantation, while others oppose the practice, deeming it a violation of shari'ah. Ashkenazi et al. [10] report that a mere 8% of the population comprises ultraorthodox Jews who adhere to a stringent interpretation of Halacha regulations, which define death as the cessation of cardiac and respiratory functions, therefore rejecting the concept of brain death. Potential donors adhere to this rationale and abstain from contributing. Ju et al. [11] report on Buddhist perspectives regarding organ donation and transplantation. Generally, most religions endorse organ donation. Nonetheless, even within a single religion, divergent agendas may be pursued. For the individual person, it is advisable to consult the pertinent religious institution with any uncertainties.

Pattinson [12] argues that, from a legal standpoint, the Human Tissue Act 2004 in the USA must recognize the ownership of organs from both living and deceased donors, challenging the prohibition on directed donation and advocating for legislative reform. In other countries, ownership is either subject to legal regulation or remains unclear, complicating the donation process and potentially hindering further testing if required.

8.4.2 Summaries of Allocation

Transparency and equity are widely recognized as the fundamental objectives of allocation. Below are some potential ways for the allocation process, presented for the reader's understanding:

- **The first-in, first-out protocol**: All patients are organized on a waitlist based on their arrival sequence. The organ offer is presented to the patient who has been awaiting the longest upon availability. If the patient is deemed non-transplantable,

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the offer is then made to the next patient in line. Consequently, the process remains unaffected by extraneous factors.

- **Allocation according to established parameters**: Field specialists are consulted before accepting additional allocation parameters. Moreover, ethical, religious, and community considerations are addressed and implemented. Priorities have been established. Patients are then added to the waitlist and enumerated according to the established criteria upon the availability of a donor organ.

8.4.3 The Distribution of the Organs

To the extent that the organs are available, the allocation process can be carried out. Because the rules are subject to change, it is extremely difficult to report or list them adequately. Across the globe, there are many organizations that are responsible for organ procurement and allocation. van Rood was the first person to establish an organ procurement and allocation organization (OPO), Eurotransplant [13]. It was and probably is also the first and only international organization, and its members include currently Austria, Belgium, Croatia, Germany, Hungary, Luxembourg, the Netherlands, and Slowenia. The kidneys were the first organs to be used in the Eurotransplant scheme. Later, the other transplantable organs were included in the allocation scheme. In the beginning, HLA matching was used to determine the allocation of the kidneys. As a result of Eurotransplant, many other organizations all around the world have been founded. These were nation-driven organizations. Subsequently, the regulations were required to be ratified by the countries that were participating.

The increase in nations with formal institutions signifies a story of achievement; nonetheless, the global objective remains unattained.

Table 8.1 distinctly illustrates the issue of global procurement and allocation. While Europe has a relatively high number of countries with official entities, Africa has a considerably lower count. This disparity also indirectly indicates the potential funding of the process by local authorities, and needs attention.

Table 8.1 Count of nations having allocation organizations.

WHO region	Number of countries	Countries with organizational information	Countries with an official organization
Africa	46	5	1
Americans	36	19	18
Easter Europeans	20	13	10
Europe	53	39	30
South East Asia	11	9	5
Western Pacific	27	13	11
Total	193	98	75

Source: Adapted from [14].

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8.5 Postmortem Allocation

8.5.1 Renal System

The kidneys are the most frequently transplanted organs globally. In countries with adequate organ and patient availability, an allocation scheme is implemented. Typically, factors, such as waiting time, defined as the date of the initial dialysis, patient immunization (sensitization) against HLA system factors, patient age, distance to the donor center, and other parameters are considered. Based on national guidelines, a crossmatch involving donor cells and recipient serum is performed prior to transplantation. This procedure is often bypassed for unsensitized patients and, in some cases, for sensitized patients, depending on local protocols. This process necessitates rigorous testing and the approval of clinicians and laboratory staff. Organs are allocated to patients selected by the algorithm, with a final crossmatch conducted at the patient's affiliated center if required.

In certain global regions, local issues necessitate resolution. For instance, in Asia, Woo [15] indicates that procuring cadaveric kidneys for renal transplantation is hindered by socio-cultural beliefs and customs. The difficulties associated with living-related kidney donation are primarily social rather than cultural, stemming from a complex web of familial pressures and personal conflicts impacting both the donor and recipient. Misconceptions, including fears of death, breaches of bodily sanctity, and religious apprehensions, result in suboptimal organ utilization compared to Western nations. Furthermore, a successful living-related program may compromise post-mortal organ procurement [16].

In Europe, various schemes focus on optimizing graft outcomes and the survival of both patients and grafts. The Eurotransplant kidney allocation system aims to reduce waiting times and enhance the survival rates of patients and grafts [17]. Lee and Abramowicz [18] discuss the application of the Kidney Donor Risk Index to improve transplantation outcomes. The importance of the immune response, especially the humoral response, has grown in the context of postmortem kidney transplantation. Specialized programs have been established to assist particularly challenging patients, such as highly sensitized individuals [19], the elderly [20], and the use of specific medications to prevent hyperacute rejection and create a therapeutic window.

The international comparison among various Organ Procurement Organizations (OPOs) facilitates the implementation of new regulatory measures, as exemplified by Israni et al. [21], who present the allocation strategy enacted in 2013. Nonetheless, the nation exhibits a higher mortality rate compared to Canada [22]. In the United States, the shortage of deceased donor kidneys has been scrutinized, leading to the development of a utility-based methodology to align donors with recipients possessing similar projected survival rates. The newly proposed framework may potentially reduce kidney allocation for candidates aged 50 and older while concurrently enhancing allocation efficiency [21]. Comparable initiatives, along with the prospective incorporation of epitope matching in the allocation process, will augment the efficacy of transplantation. However, these analyses necessitate extensive data and close collaboration among the centers.

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In other regions of the world, clinicians and laboratory members are struggling. With low access to donor organs. Therefore, the schemes concentrate on either restricted patient groups, Latin America, regarding access to deceased donor kidney transplants for pediatric patients in the region [23]. The allocation process is increasingly focused on minimizing HLA mismatches while also considering additional factors that influence post-transplant outcomes. There is a necessity for enhanced organ procurement processes, as highlighted by various authors in the study on renal replacement therapy in Latin American end-stage renal disease [24]. Similarly, in the Arab world, most countries depend on living transplants. Deceased donor programs are restricted to some countries, such as Saudi Arabia, Tunisia, Kuwait, etc. [25]. Donations remain below the levels reported in other countries. Recently, there are new programs, and DCD appears to be extremely promising. A significant issue is the elevated denial rate on donations of 43% [26].

8.5.2 Summaries and Conclusions on Renal Procurement and Allocation

Global kidney allocation adheres to stringent regulations and aims to provide each patient with an appropriate organ. However, there are numerous regional disparities. In Europe, the United States, and Canada, due to the availability of patients and donors, as well as institutional support, new allocation mechanisms have been introduced. Moreover, the compatibility indicated by the patients' existing humoral response, characterized by specific antibodies, along with superior immunosuppressive therapy, enables even highly sensitized patients to undergo successful transplantation. In areas where post-mortem transplantation is underdeveloped, living donation programs are highly successful. A scheduled procedure is feasible, and the outcomes are very encouraging. Therefore, possible usable postmortem organs are wasted. Critiquing underdeveloped programs from a Western perspective, where resources are abundant, is rather unpleasant. It is essential to offer assistance and knowledge in both procurement testing and allocation. Moreover, countries and regions with limited transplantation opportunities benefit from the definitive outcomes of others and can adequately treat their patients. Each nation and region ought to choose the approach that yields optimal outcomes for their patients.

8.5.3 Cardiac and Pulmonary Systems

The allocation of donor hearts and lungs follows criteria like those for kidneys, excluding direct HLA matching. Factors, such as body indices, weight, and age, are considered. A crossmatch prior to transplantation is essential, especially for sensitized patients. Unsensitized patients may undergo transplantation based on criteria set by the transplantation institution, as well as regional and national regulations pertinent to the patient's location. The urgency for transplantation is increasingly critical. The absence of HLA as a criterion does not imply that

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immunology is clinically insignificant; rather, the current typing methodology is excessively time-consuming, thereby extending the matching process.

A growing number of patients are provided with assist devices and mechanical support to facilitate the period leading up to transplantation. These devices may augment the number of sensitized patients on the waitlist. Numerous European Organ Procurement Organizations (OPOs) have implemented an urgency-based allocation method, incorporating a national exchange balance to address disparities in donation rates [27]. The analysis examines the implementation of a balancing approach, encompassing post-transplantation prospects and anticipated clinical outcomes for patients remaining on the waitlist [28]. Similar procedures are conducted for the lungs, such as the implementation of the Lung Allocation Score and crossmatching when deemed necessary. However, one should be cognizant that multiple transplants and domino transplants are also performed. If the recipient's lung is deemed appropriate, a double transplant is conducted, and the explanted lung is then provided to another patient on the waiting list.

Like other institutions, the US and Canada are confronting an increasing population of individuals with heart failure. The United Network for Organ Sharing (UNOS) Thoracic Committee tackled the issue by categorizing patients largely according to wait-list mortality and broadening regional limits for organ allocation. Nevertheless, obstacles persist in prioritizing specific patient populations, particularly those utilizing assistive devices [28]. Both nations prioritize the treatment of the most critically ill patients. The distribution of donor hearts and lungs is conducted according to criteria akin to those for kidneys, without HLA matching. A crossmatch before grafting is essential, particularly for sensitized patients. Unsensitized patients may be transplanted based on the criteria established by the transplantation institution, as well as the regional and national regulations applicable to the patient's location. The urgency for a transplant is becoming increasingly significant in the operation.

In other world regions, e.g. Asia, heart transplantation is crucial owing to the rising prevalence of heart failure [29, 30]. Although the treatment demonstrates effects comparable to or superior to worldwide statistics, it encounters typical problems faced by other regions, such as donor shortages, religious views, and ineffective allocation procedures [31, 32]. The rise in heart failures in Asia, along with the aging patient demographic and substantial lifestyle alterations, may require an escalation in transplantation procedures. Limited data on heart failure, in comparison to the Western world, continues to provide a challenge [32]. Similar challenges are faced in other regions, such as Latin America, as allocation policies are hindered by organ shortages and organizational issues. The implementation of assistive technologies, as previously mentioned, may provide a provisional remedy, resulting in reduced death rates [32]. The necessity for effective and equitable allocation rules is crucial, particularly due to the increasing demand for treatment throughout Latin America.

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8.5.4 Hepatic Organ

The distribution of liver resources has been enhanced by the introduction of the Model for End-Stage Liver Disease (MELD) score, which was adopted in the United States in 2002 and subsequently by other nations. Numerous amendments have since been made, with current policy changes focused on achieving fairness, equity, and improved outcomes for patients awaiting liver transplantation [33].

Regulations governing liver distribution in the United States have significantly evolved in recent decades to address the disparity between organ supply and demand. The MELDS score is currently in use and will likely be revised to accommodate the changing requirements of patients and physicians. In Canada, allocation policies for hepatocellular carcinoma may vary. The necessity for standardized allocation methods across all centers within a country or region remains contentious, as individual centers may implement their own systems.

The MELD score has been implemented, as previously indicated. National allocation organizations benefit more than their multinational counterparts, which must adhere to varying national regulations. Hepatocellular carcinoma (HCC) is a common criterion for liver transplantation, and the allocation strategies for HCC patients vary across European countries, as similarly observed in Canada. Despite the lack of a unified European allocation system, long-term survival rates for HCC patients are comparable to those recorded in the United States [34, 35].

Liver transplantation in Latin America faces considerable challenges, including inadequate financial resources, education, and organization [36]. The region performs approximately 4.4 liver transplants per million individuals annually, with Brazil, Argentina, and Uruguay leading in transplant rates [37]. However, there is a disparity among nations. Some countries have advanced transplant systems comparable to those in affluent nations, while others either lack operational programs or encounter resource limitations. The MELD system has been predominantly adopted, and to address organ shortages, improved criteria derived from kidney transplantation have been employed. Despite progress, geographic disparities in access to transplantation remain a significant obstacle, necessitating continuous efforts to enhance organ allocation and increase donor rates [34].

8.6 The Crossmatch

8.6.1 Virtual Crossmatch

Upon inputting the HLA typing findings into the primary information system, the selection of kidney recipients takes place, succeeded by a virtual crossmatch, which relies on the HLA type of the organ donor and the patient's antibody screening. This procedure lead to the definition of unacceptable incomatibilities or unacceptable mismatches. These terms are the HLA antigens and their respective alleles, which the patient recognized as non-self and made specific antibodies.

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Contrasting the acceptable mismatches [38], where the HLA antigens per patient towards which no antibodies were observed. The establishment of the virtual crossmatch reduces the workload of the laboratory during duty hours and increases the reliability of it. However, the discussion is still ongoing. Especially for patients with HLA specific antibodies towards which no direct reagents are available, because they are not included in a test kit, the decision can be doubted. Here, a wet crossmatch is required.

8.6.2 Wet Crossmatch

There are numerous discussions over the decisive crossmatch test before transplantation. This stage is considered essential for organs, such as the kidney and pancreas, as well as for sensitized heart and liver awaiting patients. Unsensitized individuals may receive the organ without a prospective crossmatch [39]. The decision rests with the pertinent transplantation center and the national authorities. If a crossmatch is necessary, a method must be chosen. Depending on the location, either a complement-dependent cytotoxicity assay (CDC) or a flow cytometry assay (FCM) may be conducted. In mainland Europe, the CDC is the leading method, while in the US and Canada, the FCM is performed. The authors of the present do not select either method since the decision is in the hands of the local or national bodies. Additional information is provided in the same issue.

A conclusive wet crossmatch is usually performed at the patient's facility. Should the kidneys be locally accessible, the wet crossmatch may be performed simultaneously with the typing.

8.7 Prospective Outlooks

The procurement and distribution of postmortem organs is a significant challenge. To ensure transparency and equity, all actions must be well documented, and, in case of need, provided to the respective national bodies or insurance companies for evaluation. Nevertheless, several procedures must be undertaken prior to an organ transplantation. The primary impediment is the patient's immunological configuration. Consequently, lifelong immunosuppressive medication must be administered, at least for most patients. The factors of the HLA system play a crucial role. Until now, matching for all organs was outside the scope due to the time required for typing. This can presently be accomplished within an acceptable timeframe via third-generation sequencing (TGS) [6, 7]. Nonetheless, this must be endorsed by the transplantation community following the assessment of pilot research. A significant advancement in transplantation is the creation of "invisible" organs [40], which, if successful, will transform the field, alongside xenotransplantation [41]. Needless to say that any changes in either procurement protocols or allocation procedures must be accepted by the local, national, or international bodies.

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9

Basic Concepts of Organ Rejection

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9.1 Introduction

The concept of self versus foreign is fundamental to immunology, defining how the immune system distinguishes between the body's own tissues and foreign entities such as pathogens, allergens, transplanted organs, or artificial materials. This distinction forms the basis for immune tolerance and immune responses. While the immune system's ability to recognize self and foreign antigens is essential for protecting the body from infections and foreign invaders, it can also lead to pathological conditions such as transplant rejection, autoimmune diseases, and cancer.

9.2 The Immune System: Balancing Self and Foreign Recognition

As seen in this book, the immune system consists of a complex network of cells, tissues, and molecules that work together to protect the body from harmful invaders such as pathogens and foreign particles. It is broadly divided into two components:

- **Innate immunity:** The first line of defense, which responds to general patterns of infection and is non-specific.
- **Adaptive immunity:** A more specialized immune response that involves T and B lymphocytes, which can recognize specific antigens and generate long-lasting memory.

A fundamental aspect of adaptive immunity is its ability to recognize and respond to foreign antigens while maintaining self-tolerance to avoid attacking the body's own cells. The recognition of foreign antigens is central to the immune response, while tolerance to self is crucial for maintaining health and preventing autoimmunity [1, 2].

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9.3 Antigens: Self and Foreign?

Antigens are the molecules that trigger an immune response. They can be either self-antigens (those derived from the body) or foreign antigens (such as those from pathogens or transplanted tissues). Antigens are typically presented to the immune system by the specialized cells known as antigen-presenting cells (APCs). These APCs, such as dendritic cells, macrophages, and B cells, process antigens and present them on their surface using major histocompatibility complex (MHC; human leukocyte antigen (HLA)) molecules.

- **MHC class I molecules** present antigens from intracellular proteins (both self and foreign) to $CD8^+$ cytotoxic T cells.
- **MHC class II molecules** present antigens from extracellular sources to **$CD4^+$ helper T cells**.

This system allows the immune system to recognize and respond to foreign pathogens while preventing the immune system from attacking self-tissues. In the case of transplantation, the HLA-proteins themselves become prone to being an antigen apart from their presented peptides [3]. Due to their very high polymorphicity, the proteins of the recipient rarely have the same amino acid sequence as the donor. Therefore, the compatibility of donor and recipient needs to be carefully evaluated using techniques and knowledge of histocompatibility and immune genetics in order to facilitate a successful transplantation. Even in the case of a full match of the HLA alleles between recipient and donor, there are still many more differing proteins left that can lead to rejections. Even in the case of identical siblings, rejection can occur and needs to be dealt with. How to deal with the, in most cases, fatal reactions of rejection, the next chapter will show.

9.4 Immune Tolerance: Maintaining the Balance

The immune system's ability to distinguish between self and foreign is maintained through immune tolerance, a critical mechanism that prevents the body from attacking its own cells. Tolerance is developed in two phases: central tolerance and peripheral tolerance.

9.4.1 Central Tolerance

Central tolerance occurs during the development of immune cells, particularly in the thymus for T cells and the bone marrow for B cells. During this process, immune cells that strongly recognize self-antigens undergo negative selection, effectively being deleted or rendered nonfunctional. This ensures that most self-reactive cells are removed before they can attack the body.

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9.4.2 Peripheral Tolerance

Some self-reactive immune cells may escape the central tolerance process and enter the periphery. Peripheral tolerance mechanisms include:

- **Anergy**: Immune cells that encounter self-antigens without proper co-stimulation become non-responsive.
- **Regulatory T cells (Tregs)**: Specialized T cells that suppress autoreactive immune responses, maintaining immune homeostasis.

Together, central and peripheral tolerance mechanisms ensure that the immune system is capable of distinguishing self from foreign, minimizing the risk of autoimmune reactions [4, 5]. Understanding tolerance also is crucial for developing new drugs to prevent rejection or help the body to fight cancer cells successfully.

9.5 Autoimmunity

Autoimmunity arises when the immune system loses its ability to distinguish itself from foreign antigens. This breakdown of self-tolerance leads to the immune system attacking the body's own tissues. Autoimmune diseases can arise due to a combination of genetic predisposition and environmental triggers such as infections or hormonal changes. Examples of autoimmune diseases are:

- **Rheumatoid arthritis**: In which T cells attack the joints.
- **Type 1 diabetes**: In which T cells destroy insulin-producing pancreatic beta cells.
- **Multiple sclerosis**: In which the immune system attacks the nervous system.

These diseases are often characterized by the presence of autoantibodies and autoreactive T cells that target self-antigens. In many of these autoimmune reactions, there are special HLA alleles involved that can interact either with distinctive protein constellations of a person (see chapter about HLA disease associations) or drugs (see chapter about drug associations) [6, 7].

9.6 Foreign Recognition Leading to Rejection

9.6.1 The Role of Dendritic Cells in Rejection

Dendritic cells (DCs) are the most potent APCs and play a central role in activating T cells during rejection. In the context of transplantation, DCs can migrate from the transplanted organ to the lymph nodes, where they present donor antigens to naïve T cells. DCs may exist in two different states [8]:

- **Immature dendritic cells:** These cells are responsible for capturing and processing antigens from the transplanted tissue.
- **Mature dendritic cells:** Once activated, they present processed antigens to T cells, initiating the rejection process.

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9.6.2 The Cellular Mechanisms of Rejection

Rejection is primarily driven by immune cells that recognize and attack foreign tissue. Two main types of immune responses are involved in rejection: **indirect recognition** and **direct recognition**.

9.6.2.1 Indirect Recognition

In this mechanism, recipient APCs process donor antigens and present them to T cells via MHC molecules, triggering an immune response. This is generally slower but can be more persistent and contribute to chronic rejection [9].

- **Cytotoxic T lymphocytes:** These cells recognize foreign MHC I (HLA class I) molecules on the transplanted organ and kill target cells.
- **Helper T cells:** Activated helper T cells release cytokines that recruit other immune cells, such as macrophages and neutrophils, which contribute to inflammation.

9.6.2.2 Direct Recognition

This occurs when recipient T cells directly interact with donor MHC molecules present in the transplanted tissue. This leads to the activation of $CD8^+$ cytotoxic T cells and $CD4^+$ helper T cells, which initiate instant immune responses, as

- **Hyperacute rejection**: Immediate rejection, often due to pre-existing antibodies against the donor's antigens.
- **Acute rejection**: Mediated by T cells and occurs within weeks of transplantation.

9.6.3 The Role of Antibodies in Rejection

In addition to T-cell responses, antibodies play a key role in organ rejection, especially in hyperacute rejection and antibody-mediated rejection. Antibodies can recognize foreign antigens on the donor organ, especially those in the MHC and blood group antigens, and bind to them, leading to complement activation and cell death [10].

Antibody-mediated rejection

- **Preformed antibodies:** In cases of prior sensitization (e.g. previous transplants or blood transfusions), the recipient may have antibodies against donor antigens.
- **De novo antibodies:** These can develop post-transplantation due to ongoing immune responses.

That leads to chronic rejection, a long-term process that causes slow damage to the transplanted organ.

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9.7 GvHD

Graft-versus-host disease (GvHD) is a significant complication in both organ and stem cell transplantation, where transplanted immune cells recognize the recipient's tissues as foreign and in turn, mount an immune response. The normal role of the immune rejection partners is turned around.

While GvHD can occur in organ transplantation, where transplanted immune cells in the donor organ attack the recipient's tissue, it is most important in stem cell transplantation, particularly in allogeneic hematopoietic stem cell transplantation (HSCT). In this context, GvHD arises when donor-derived immune cells attack the recipient's tissues.

The underlying mechanisms involve the activation of donor T cells, which recognize recipient antigens as foreign, initiating an inflammatory cascade (Figure 9.1). Risk factors for GvHD in stem cell transplantation include the degree of HLA mismatch, the age and immune status of the recipient, as well as the source of stem cells (e.g. bone marrow, peripheral blood, or cord blood). Advances in immunosuppressive therapies and strategies to minimize GvHD, such as T-cell depletion and the use of targeted biologics, have improved outcomes. Yet GvHD remains a major cause of morbidity and mortality in HSCT patients [11, 12].

9.8 Conclusion

The self-foreign concept is central to understanding the immune system's complex mechanisms of defense and tolerance. Whether defending against pathogens, maintaining self-tolerance, or responding to transplanted organs, the immune system's ability to recognize self from foreign is crucial for maintaining health. Disruptions to this delicate balance can lead to autoimmune diseases, cancer, and transplant rejection. Gaining insight into these mechanisms may lead to therapeutic approaches that enhance immune responses in immunodeficiency or cancer, while also helping to prevent transplant rejection and autoimmune diseases. Transplantation presents a unique challenge in the self-foreign concept. When an organ or tissue is transplanted from one individual (donor) to another (recipient), the recipient's immune system must differentiate between its own tissues (self) and the foreign tissue of the transplanted organ. This recognition leads in most cases to rejection, where the immune system mounts an attack against the transplanted tissue.

More information on handling rejection will be provided in the next chapter. Generally, immunosuppressive drugs are used to dampen the immune response, but these can increase the risk of infection and malignancy. So, there is a need for newer drugs that overcome these side effects, save the organ, and don't threaten the health of the recipient.

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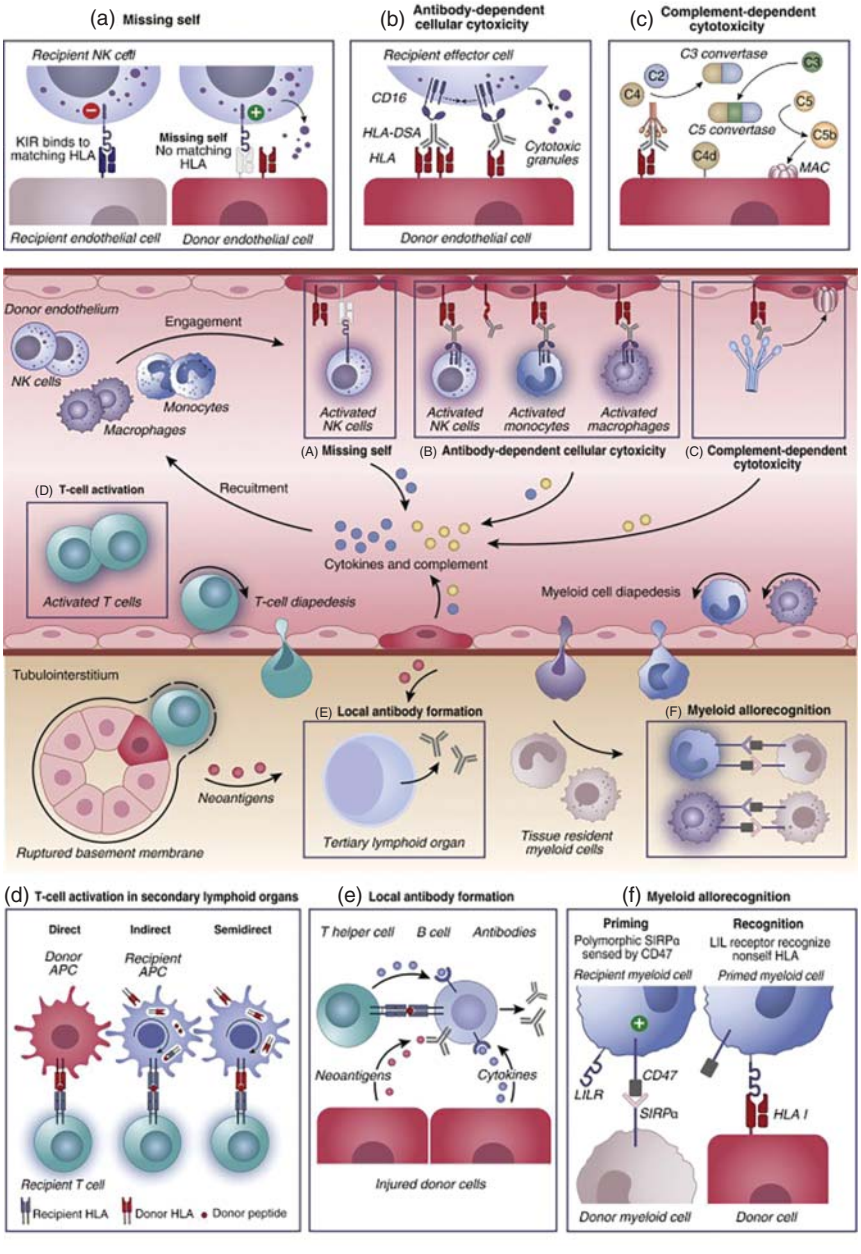

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Figure 9.1 Mechanisms of allorecognition in kidney transplantation. Circulating recipient-derived immune cells may engage or transgress the donor endothelium. Subsequent immune cell activation and graft injury cause the release of cytokines and complement, perpetuating the inflammatory response. (a) Recipient natural killer (NK) cells are disinhibited when a killer cell Ig-like receptor (KIR)–specific class I human leukocyte antigen (HLA) is not detected on the donor target membrane. (b) NK cells, monocytes, and macrophages bind the constant fraction of anti-HLA donor-specific antibodies (HLA-DSA) that are engaging with donor HLA molecules, resulting in cellular activation and release of cytotoxic granules. (c) C1qrs engagement to HLA-DSA triggers classic complement pathway activation, resulting in the formation of the membrane attack complex (MAC) on the donor endothelium. C4d, a by-product of the complement cascade, forms a covalent bond with the donor endothelium. (d) T-cell activation in secondary lymphoid organs occurs through cognate interaction with donor HLA molecules on migrated donor antigen-presenting cells (APCs; direct allorecognition), processed donor HLA displayed by recipient APCs (indirect allorecognition), and intact donor HLA molecules on recipient APCs through cross-presentation (semidirect allorecognition). (e) Exposure of neoantigens following graft injury may lead to local activation of T and B cells, leading to the intragraft production of antibodies. (f) Recipient monocytes are primed by increased binding strength of CD47 with allogeneic signal regulatory protein alpha (SIRPα). Primed myeloid cells engage with mismatched HLA class I molecules through leukocyte Ig-like receptors (LILRs), leading to proliferation and increased responsiveness. Source: Callemeyn et al. [11]/with permission of Elsevier.

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10

Treatment of Organ Rejection

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10.1 Introduction

The T lymphocyte is considered the main effector in allograft rejection. Allorecognition (the process by which T lymphocytes recognize the allograft as foreign) is stooled on the activation of T lymphocytes by antigen-presenting cells (APCs). APCs (dendritic cells, macrophages, B lymphocytes, and activated endothelial cells) activate T lymphocytes by presenting allo-antigenic peptides such as donor HLA either in a direct, semidirect or indirect way. This complex of the antigenic peptide and the MHC proteins on the APCs is recognized by the T-cell receptor (TCR). T-cell activation is currently explained by the "three-signal model" (Figure 10.1). The first signal describes the interaction between the T-cell receptor and donor antigen peptides bound to MHC molecules on APCs. Proper T-cell activation can only occur in the advent of a second signal, which implies co-stimulation through interaction between accessory ligands and receptors on APCs and T lymphocytes. The most important co-stimulatory signals are the interaction between CD28 on all naive T lymphocytes and B7.1 (CD80) and B7.2 (CD86) on APCs, CD 154 (or CD40 ligand) on activated $CD4^+$ T lymphocytes and CD40 on APCs, T lymphocyte integrins LFA-1 and CD2 (LFA-3), and ICAM-1/ICAM-2 and CD58 on dendritic cells (DCs). Signal 1 and 2 lead to T lymphocyte activation and proliferation. Cytokines secreted by APCs and activated T lymphocytes further enhance T-cell activation and determine further T-cell differentiation into specialized T lymphocyte subsets (Signal 3).

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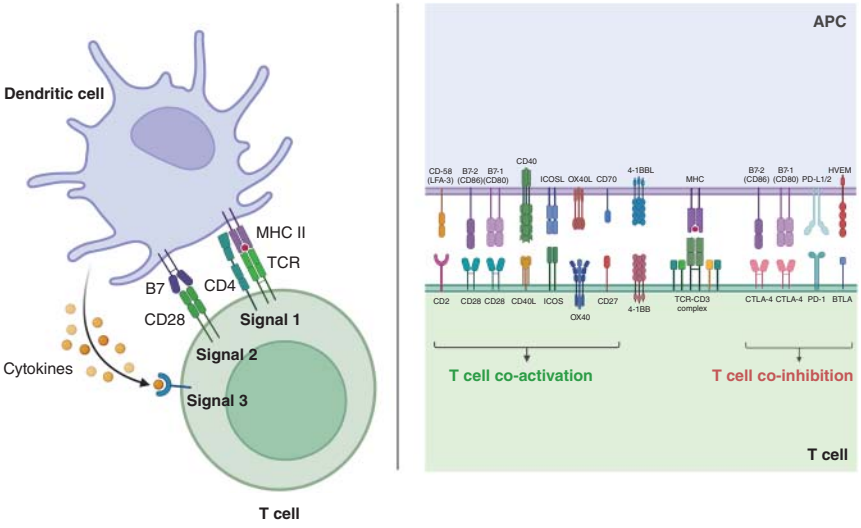

Figure 10.1 Three-signal model of allorecognition. Signal 1 implies the interaction between the TCR and antigen presented by MHC I or II molecules on antigen-presenting cells. Signal 2 describes the co-stimulatory signals necessary for T-cell activation. A non-exhaustive overview of the most well-known co-stimulatory and co-inhibiting interactions is described on the right-hand panel. Signals 1 and 2 lead to T cell activation, after which cytokines secreted by both APCs and T cells lead to further T cell activation and differentiation. Created in BioRender.com.

10.2 Prevention of Rejection: Induction and Maintenance Immunosuppression

All current transplant immunosuppressive regimens are primarily aimed at sufficiently suppressing T lymphocyte allorecognition. This comes with the cost of an increased risk of infection and, with time, also malignancy. Balancing these risks against the risk of allograft rejection has become more and more important with current highly effective regimens permitting an increasingly long allograft survival.

10.2.1 Drugs

10.2.1.1 Corticosteroids

After being discovered in the 1940s, glucocorticoids rapidly became one of the mainstays of transplant immunosuppression. Prednisolone and methylprednisolone are the molecules most commonly used. They have pleiotropic effects on the immune system but most importantly inhibit T-cell activation and cytokine expression. Glucocorticoids bind to cytosolic glucocorticoid receptors (GR), which are normally in an inactive state due to their binding to cytoplasmic chaperone complexes such as heat shock proteins and immunophilins. Almost every cell type throughout the body expresses GR. As glucocorticoids are lipid soluble, they diffuse through the cell membrane, and upon binding of glucocorticoids to their GRs in the cytosol,

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the GR dissociates from its chaperone and translocates to the cell nucleus. Binding on specific DNA regions called "glucocorticoid responsive elements" (GREs) results in suppression of the transcription of numerous genes implicated in T-cell activation and proliferation. The glucocorticoid-GR complex also inhibits the binding of key transcription factors nuclear factor κB and activator protein-1 to their target DNA sequences [1].

Because of the universal expression of glucocorticoid receptors throughout the body, glucocorticoid therapy is associated with numerous and diverse adverse effects. Aside from the increased risk of infection and malignancy associated with all immunosuppressive drugs, corticosteroids are associated with an increased risk of osteoporosis, osteonecrosis, increased appetite and weight gain, glucose intolerance and diabetes mellitus, glucocorticoid myopathy, cataract, hypertension, impaired wound healing, a range of psychopathological effects (ranging from euphoria and psychosis to depression), and several cosmetic side effects such as acne, increased skin fragility, striae, and hirsutism.

The most commonly used preparations are prednisolone and methylprednisolone. They are a standard element of pre-transplant induction regimens as well as maintenance immunosuppressive regimens after transplantation, although increasing efforts are being made for early corticosteroid withdrawal or corticosteroid-free regimens [2].

10.2.1.2 Calcineurin Inhibitors (CNIs)

Since the isolation of cyclosporin from the fungus *Tolypocladium inflatum* in the 1970s and its clinical introduction in the field of transplantation in the 1980s, CNIs have revolutionized the field of transplantation due to an important decrease in rejection rates and improved graft survival. They continue to be the backbone of current immunosuppression in solid-organ transplantation. Tacrolimus (also called FK506) was isolated 10 years later from the fungus *Streptomyces tsukubaensis* and is currently the most frequently used CNI due to its higher potency and a somewhat more attractive side-effect profile for most people.

Both cyclosporine and tacrolimus exert a powerful inhibitory effect on T-cell activation through interference with the calcineurin-NFAT pathway (Figure 10.2). Upon binding of the TCR with the alloantigen presented by MHC molecules on APCs, an influx of intracellular calcium leads to calcium binding to intracellular calmodulin. The Ca-calmodulin complex activates cytoplasmic calcineurin, which is a calmodulin-dependent serine/threonine phosphatase. Once activated, the calmodulin-calcineurin complex dephosphorylates and activates cytoplasmic nuclear factor of activated T cells (NFAT), leading to its translocation to the cell nucleus where NFAT functions as a transcription factor of several key genes in T-cell activation such as IL-2, IL-4, IFN-γ, and TNF-α. Upon binding of cyclosporin and tacrolimus with cytoplasmic immunophilins (cyclophilins and FK-binding proteins, respectively), these complexes bind and inhibit calcineurin and prevent this critical pathway in T-cell activation [3].

The most important side effect of CNIs is their nephrotoxicity, sometimes even leading to end-stage kidney disease. CNI-mediated nephrotoxicity is thought to

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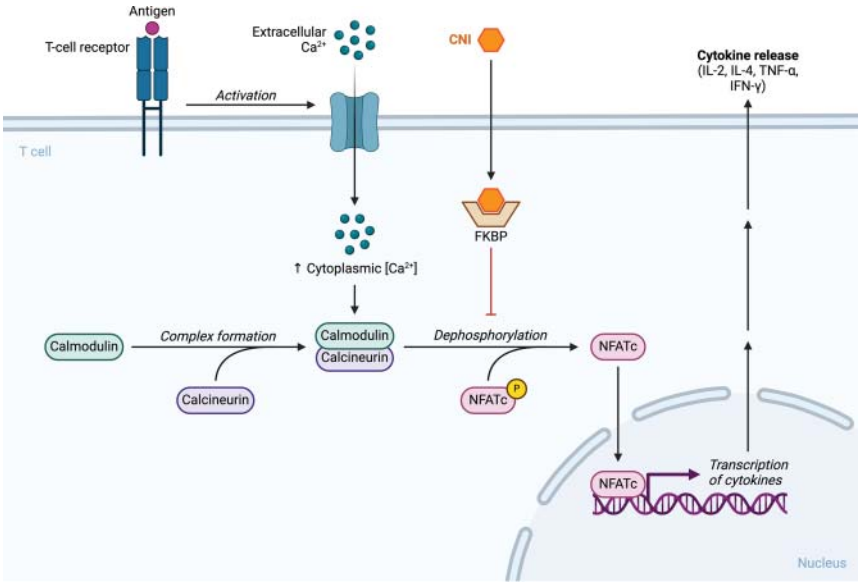

Figure 10.2 The calcineurin pathway. Created in BioRender.com.

be mediated by a dose-related vasoconstriction of the afferent renal arteriole and progressive interstitial fibrosis partly mediated by an increased expression of TGF-β [4]. Besides nephrotoxicity, CNIs are associated with hypertension, hyperkalemia, hypomagnesemia, hyperuricemia, thrombotic microangiopathy (TMA), hyperlipidemia, pancreatic islet cell toxicity leading to diabetes mellitus, neurotoxicity (presenting as tremor, dysesthesias, and cognitive difficulties), hyperlipidemia, cosmetic effects (gingival hyperplasia, hirsutism, and alopecia), and gastrointestinal side effects (hepatic dysfunction, cholelithiasis, anorexia, nausea, vomiting, and diarrhea). There are discrete differences between the side effect profile of the two CNIs, with tacrolimus being associated with higher rates of diabetes and neurotoxicity, whereas cyclosporin is more commonly associated with hypertension, hyperuricemia, hyperlipidemia, gingival hyperplasia, and hirsutism.

As these drugs are substrates of the intestinal pump P-glycoprotein and the hepatic cytochrome P450 enzyme system (most importantly CYP3A4 and CYP3A5), they are prone to important drug-drug interactions. They both have a narrow therapeutic window, and trough levels are routinely measured to prevent excessive toxicity and ensure effectiveness [5]. Target trough levels vary somewhat according to the type of solid organ transplantation and the timing of transplantation (higher trough levels in the early post-transplant period) but are generally 5–15 ng/ml for tacrolimus.

10.2.1.3 Antimetabolites: Mycophenolic acid (MPA) and Azathioprine (AZT)

Used as a common adjunct immunosuppressant together with CNIs, mycophenolic acid and azathioprine inhibit the proliferation of T and B lymphocytes by interfering with DNA synthesis.

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Azathioprine (AZT) was the first antimetabolite to be developed and used as an immunosuppressant in transplantation medicine. It saw the light at the end of the 1950s and rapidly became part of the standard transplant immunosuppressive regimen combined with corticosteroids. After the development of CNIs in the 1980s and MPA in the 1990s [6], azathioprine is currently only rarely used. It still has a role as an immunosuppressant in pregnancy as an alternative to the teratogenic drug MPA and in low-cost settings.

Azathioprine is the imidazole derivative of 6-mercaptopurine and functions as a prodrug. After intestinal absorption, it is converted to 6-mercaptopurine through reduction by glutathione transferases. It is metabolized further and conjugated with a ribose molecule to form 6-thioinosine monophosphate (TIMP) and 6-thioguanosine monophosphate (6-TGMP). 6-TGMP is further converted to the triphosphate deoxythionucleotide TdGTP, acting as a non-functional analogue of deoxyguanosine triphosphate (dGTP), blocking DNA synthesis and thus cell division (see Figure 10.3). AZT also exerts a specific inhibitory effect on *de novo* purine synthesis. Based on these mechanisms, AZT prevents rejection through its general myelosuppressive effect, leading to a reduction of circulating monocytes and its more specific inhibition of T and B lymphocytes, which rely solely on *de novo* purine synthesis, whereas other cell types can also make use of the purine salvage pathway.

The main side effects are dose-related bone marrow suppression and leukopenia. This risk is dramatically increased in case of low thiopurine methyltransferase (TPMT) activity or when combined with the xanthine oxidase inhibitor allopurinol. There is also a risk of cholestasis, hepatitis, and veno-occlusive disease.

When used as an adjunct to CNI therapy, the dose is 1–2 mg/kg with no need for therapeutic drug monitoring. Leukocyte count and transaminase/bilirubin levels should be monitored after initiation and dose adjustment.

Mycophenolic acid (MPA) is a mycotoxin derived from several *Penicillium* species. It is sold as mycophenolate mofetil (MMF, Cellcept®), the 2-morfolinoethyl ester of MPA, a prodrug developed to ameliorate bioavailability. Enteric-coated mycophenolate sodium (Myfortic®) has also been developed to ameliorate gastrointestinal side effects commonly observed with MPA. MMF is converted to MPA by intestinal, plasma, and hepatic esterase, after which it inhibits the enzyme inosine monophosphate dehydrogenase (IMDPH). IMDPH is a rate-limiting enzyme in *de novo* purine synthesis as it catalyzes the formation of guanosine monophosphate (GMP) from inosine monophosphate (IMP). Depletion of guanosine nucleotides (GMP, GTP, dGTP) inhibits DNA synthesis and cell division. It preferentially inhibits B and T lymphocytes as they are dependent on the *de novo* purine synthesis, whereas other cell types can also make use of the purine salvage pathway catalyzed by hypoxanthine-guanine phosphoribosyltransferase. Furthermore, it has a strong affinity for the type II isoform of IMDPH, which is expressed in activated B and T cells, whereas the type I isoform present in most cell types is inhibited with much less potency.

The most important adverse effect of MPA is its gastrointestinal intolerance with diarrhea, bloating, nausea, and vomiting. Dose reduction or dispersion into three

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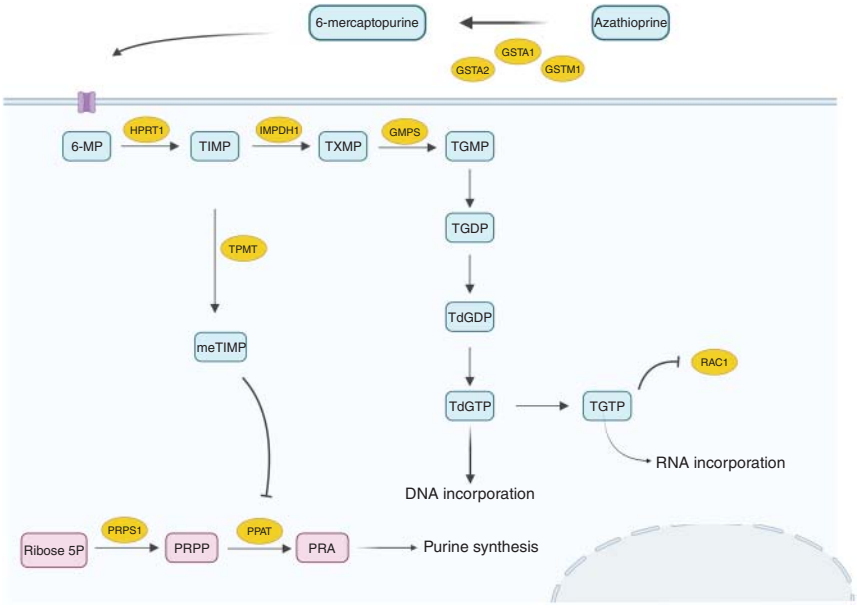

Figure 10.3 Mechanism of action of azathioprine. After conversion of AZT to 6-MP by glutathione transferases, it is taken up intracellularly through several transmembrane channels (SLC28A2, SLC28A3, SLC29A1, and SLC29A2). It is then converted into thioinosine monophosphate (TIMP) by hypoxanthine guanine phosphoribosyl transferase (HPRT1) with 5-phospho-D-ribose-1-pyrophosphate (PRPP) as the phosphoribosyl donor. TIMP is then converted into thioxanthosine monophosphate (TXMP) by inositol monophosphate dehydrogenase, after which TGMP (thioguanosine monophosphate) is formed by guanosine monophosphate synthetase (GMPS). TGMP is converted into thioguanine nucleotide diphosphates and triphosphates. TGTP (6-thio-guanosine triphosphate) is incorporated into RNA, whereas TdGTP (6-thio-deoxy-guanosine triphosphate) is incorporated into DNA. The latter leads to impaired DNA replication, single-strand DNA breaks, and impaired DNA repair, ultimately leading to cell toxicity. AZT also leads to the formation of methylmercaptopurine nucleotides, which directly inhibit de novo purine synthesis mediated by the enzymes PRPS1 (phosphoribosyl pyrophosphate synthetase 1) and PPAT (phosphoribosyl pyrophosphate amidotransferase). Furthermore, TGTP directly inhibits Rac1 (ras-related C3 botulinum toxin substrate 1), a small Rho GTPase regulating cell proliferation. Inhibition of Rac1 leads to T lymphocyte apoptosis. Other abbreviations used: PRA, 5-phosphoribosylamine; PRPP, 5-phospho-D-ribose-1-pyrophosphate). Created in BioRender.com.

to four gifts across the day ameliorates these symptoms rapidly. When continued despite ongoing diarrhea, MPA can lead to an inflammatory colitis [7]. The second most important side effect is bone marrow suppression with leukopenia, anemia, and thrombopenia. It is a known teratogenic drug with an increased risk of spontaneous abortions and congenital malformations. Women of childbearing age should be counseled regarding the absolute need for contraception, and MPA should be stopped six weeks prior to conception, during pregnancy, and lactation.

MPA (MMF) is rapidly absorbed with a peak concentration at one hour after ingestion and a half-life of 17.9 ± 6.5 hours. MPA is glucuronidated to the inactive

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metabolite MPAG, which is partly excreted in the bile via OATP transporters, where it can serve as a substrate for enterohepatic recycling through deglucuronidation by gut bacteria and re-uptake as MPA. This leads to a second absorption peak at 6–12 hours after administration. It is mainly excreted by the kidneys as MPAG. Routine therapeutic drug monitoring is not performed, and trough levels have been shown to be inconsistent and unreliable in assessing drug exposure. In some cases, an "area under the curve" or AUC is calculated based on repetitive sampling. This was initially performed through repetitive drug sampling during the first 12 hours after ingestion, but abbreviated two- and three-sample models are now being used [8, 9]. An AUC of 30–60 μg·h/ml is recommended as levels < 30 μg·h/ml have been associated with an increased risk of rejection [10].

10.2.1.4 mTOR Inhibitors

The enzyme mTOR (mammalian target of rapamycin) is a protein kinase with crucial regulatory functions in cell growth and survival. In the 1970s, a macrolide with antifungal activity was isolated from the soil bacterium *Streptomyces hygroscopicus* during an expedition to Easter Island (also called Rapa Nui), which led to the name Rapamycin (later renamed sirolimus). Rapamycin was shown to inhibit the cell cycle at the G1 to S transition in T lymphocytes, ultimately leading to its FDA approval for use in kidney transplantation at the end of the 1990s. Everolimus was subsequently developed in an attempt to ameliorate the pharmacokinetic properties of sirolimus. It only gained FDA approval in 2010 for its use in kidney transplantation.

Both mTOR inhibitors bind to the cytoplasmic immunophilin FK506-binding protein 12 (FKBP12). This is the same immunophilin that binds tacrolimus, though binding of the mTOR inhibitors leads to a different effect. The FKBP12-bound mTOR inhibitors bind and inhibit mTORC1 (see Figure 10.4). This leads to inhibition of the maturation and differentiation of antigen-presenting cells and inhibition of T-cell activation and proliferation with a shift of T-cell fate toward an increase in T_{reg} cells, which further promotes graft tolerance. B lymphocyte activation and proliferation are also suppressed [11].

As mTORC1 is a key regulator of cell metabolism, growth, and survival throughout the body, these drugs are associated with a wide array of side effects. Just like MPA and AZT, they lead to dose-related bone marrow suppression. They are much less nephrotoxic than CNIs but are presumed to potentiate the nephrotoxic effects of CNIs when administered together. They are frequently associated with proteinuria in kidney transplant recipients in a dose-dependent manner [12]. This is partly believed to be a consequence of the discontinuation of CNIs, which are known to be antiproteinuric. Nevertheless, proteinuria has also been documented in other solid-organ transplant recipients [13]. The exact mechanism of the mTORI-associated proteinuria is not entirely understood, though direct toxic effects on the podocyte have been postulated [14]. The mTOR pathway has also been shown to mediate protective podocyte hypertrophy in experimental models of acute podocyte stress [15]. As VEGF signaling makes use of the mTORC1 pathway, mTOR inhibitors have also been associated with TMA [16]. Other side

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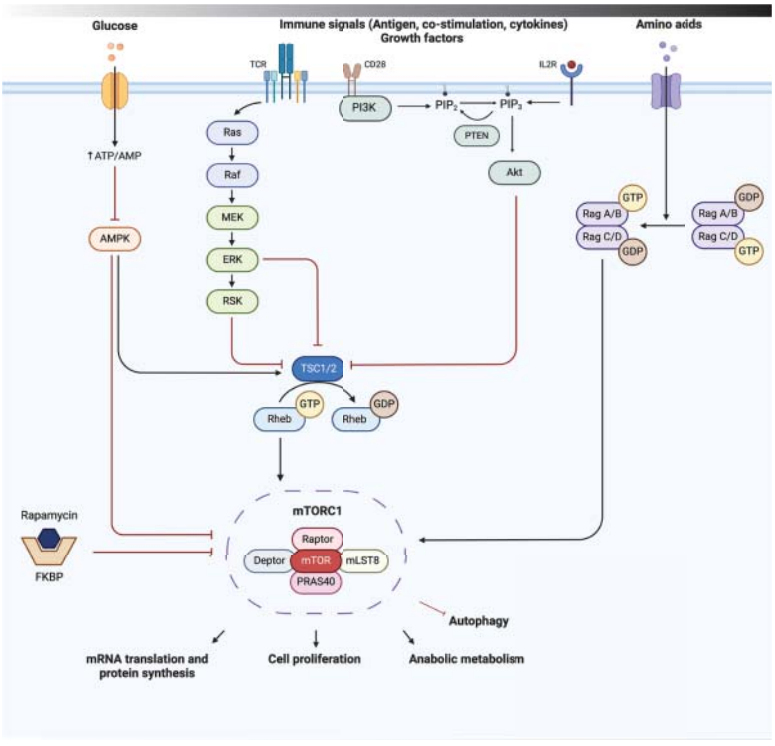

Figure 10.4 The mTOR pathway. The mammalian target of rapamycin (mTOR) enzyme plays a key role in the regulation of cell metabolism, growth, and survival as it integrates signals from multiple important signal transduction pathways implicated in both sensing of energy and nutrients and response to growth factors. General growth factors can be insulin or insulin-like growth factor, for example. T-cell-specific stimulatory signals are TCR activation by MHC-presented antigen (signal 1), co-stimulatory signals (signal 2, for example, CD28), and cytokines (signal 3, for example, IL2). mTOR is a 289 kDa serine/threonine protein kinase that resides in the cytoplasm as two distinct multimeric complexes, mTORC1 and mTORC2. mTORC1 is inhibited by rapamycin, whereas mTORC2 is only inhibited after prolonged exposure. mTORC1 is composed of mTOR, the scaffolding protein RAPTOR (regulatory associated protein of mTOR), mLST8 (mammalian lethal with SEC13 protein 8, also known as GβL), DEPTOR (DEP-domain-containing mTOR-interacting protein), and PRAS40 (the 40 kDa proline-rich Akt substrate). Both Deptor and PRRAS40 act as endogenous inhibitors of mTORC1. mTORC1 stimulates cell proliferation and growth through downstream anabolic effects on cell metabolism (stimulation of protein synthesis, RNA and nucleotide synthesis, lipid synthesis, and ATP production) and inhibition of autophagy. mTORC1 is regulated by the Rheb and Rag GTPases. The tuberous sclerosis complex (TSC), which is made up of TSC1, TSC2, and TBC1D7, acts upstream of lysosomal Rheb and converts the active GTP-bound Rheb to the inactive GDP-bound form, thereby inhibiting mTORC1. TSC is a central regulator of mTORC1 and is negatively regulated by the PI3K-Akt pathway, as well as the Ras/raf/MEK/ERK pathway, among others, such as the Wnt and TNF pathways. T-cell activation via the three-signal model leads to activation of both the Akt and ERK pathways, both leading to mTORC1 activation and enhanced T-cell proliferation. The Rag heterodimer recruits mTORC1 to the lysosome to be activated by GTP-Rheb. The Rags are lysosome-bound heterodimers, which are active when Rag A/B is GTP-bound and Rag C/D are bound to GDP. These Rags are active under amino-acid-rich conditions, regulated by several amino-acid sensors such as GATOR1, Sestrin2, CASTOR1, and SLC38A9. A third important mechanism of mTORC1 regulation is through AMPK, which is activated in situations of low cellular ATP. It acts as a master inhibitor of energy-consuming cellular processes in times of energy or oxygen shortage. AMPK will stimulate TSC and thus inhibit downstream mTORC1 activation. Created in BioRender.com.

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effects are impaired wound healing, lymphedema, mouth ulcers and stomatitis, hyperlipidemia, interstitial pneumonitis, and teratogenicity.

As mTOR inhibitors are cytostatic drugs, they have been extensively studied for the treatment of cancer. They are approved by the FDA for the treatment of advanced renal cell carcinoma, advanced hormone receptor-positive HER2-negative breast cancer, and certain types of neuroendocrine tumors. They have also been shown to reduce the risk of post-transplant malignancy [17]. In light of this, mTOR inhibitors are considered the preferential immunosuppressants in case of post-transplant malignancy. Their anti-neoplastic benefit is most evident in the case of Kaposi sarcoma [18] and post-transplant skin cancer, most importantly non-melanoma skin cancer [19, 20].

They are routinely prescribed in solid organ transplantation in CNI-sparing regimens where they are used as an adjunct to CNIs instead of MPA/AZT. As these regimens permit lower CNI exposure, they are associated with better long-term renal outcomes across most types of solid organ transplantations [21]. CNI-free regimens, usually containing an mTOR inhibitor and MPA +/− corticosteroids, remain controversial as they are associated with an increased risk of acute antibody-mediated rejection in both kidney, heart, and liver transplantation [22–25].

Just like CNIs, mTOR inhibitors are substrates of the CYP3A4 and P-glycoprotein. Sirolimus has a long half-life of 62 hours, whereas that of everolimus is shorter (30 hours). Therapeutic drug monitoring is routinely performed with target trough levels of 5–15 ng/ml for sirolimus and 3–8 ng/ml for everolimus.

10.2.2 Biologicals

10.2.2.1 Introduction

The term "biological" drug is used to describe drugs that are derived from large-scale cell culture or human blood and plasma. In transplantation, these immunosuppressive drugs are predominantly monoclonal and polyclonal antibodies that can be used for induction and treatment of rejection. Antibody therapy used for induction is classified according to the effect on circulating T lymphocytes into lymphocyte-depleting agents (ATG and anti-CD52) and non-depleting agents such as basiliximab. Other biological therapeutics routinely used in transplantation are intravenous immunoglobulins (IVIG), which are known for their immunomodulatory effects and are prescribed during induction therapy for high-risk transplantation and to treat antibody-mediated rejection. B-cell directed therapy (rituximab) is mainly used in desensitization protocols for HLA- or ABO-incompatible transplantation. Belatacept is the only biological drug currently approved for maintenance immunosuppression and is typically used as an alternative to CNIs.

10.2.2.2 Antithymocyte Globulin (ATG)

Rabbit antithymocyte globulin (rATG, Thymoglobulin®) is a polyclonal antibody preparation derived from pathogen-free rabbits immunized with human thymus tissue. These cytotoxic antibodies target various T-cell markers, leading to the

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rapid destruction of T cells (both circulating and residing in secondary lymphoid organs). They have also been shown to promote the expansion of $CD4^+$ regulatory T cells, which promote allograft tolerance [26, 27] and, when given perioperatively, reduce the risk of delayed graft function (DGF). ATG has proven to be effective in preventing and treating acute cellular rejection following renal transplantation and has also been widely used for similar purposes in heart, lung, and liver transplants. Equine ATG (eATG, Atgam®) is also available, though not routinely used in the field of solid-organ transplantation.

The use of ATG leads to a rapid and profound dose-dependent lymphopenia with low $CD4^+$ lymphocyte counts that can persist for up to several years [28, 29]. Since ATG also contains antibodies directed at other cell types (among which are B cells, NK cells, thrombocytes, and erythrocytes), other types of cytopenia can occur, most frequently thrombocytopenia and neutropenia. Since ATG is a non-humanized 100% animal-derived protein product, infusion reactions are frequent, usually consisting of fever, chills, and rarely anaphylaxis. These reactions occur typically during the first or second infusion. More serious infusion reactions are due to cytokine release syndrome (CRS), where rapid lysis of the T lymphocyte pool leads to a sudden increase in pro-inflammatory cytokines such as IFN-γ and TNF-α. CRS can lead to fever, chills, headache, rash, hyper- or hypotension, nausea/vomiting, diarrhea, arthralgias, dyspnea, and rarely, life-threatening cardiorespiratory dysfunction. To minimize these reactions, premedication with antipyretics, antihistamines, and glucocorticoids is recommended prior to administration, and the infusion is started very slowly. Rarely, serum sickness can develop one to two weeks after infusion, which usually responds well to increased doses of corticosteroids.

The optimal cumulative dosage of ATG is not clearly established, but it is likely within the range of 3–6 mg/kg [30]. Higher doses and extended treatment durations are associated with an increased risk of infection and an increased risk of developing post-transplant lymphoproliferative disease [31–33].

10.2.2.3 Anti-CD52

Alemtuzumab (or Campath-1) is a humanized monoclonal antibody targeting CD52, which is expressed on the surface of T- and B-lymphocytes, NK cells, and monocytes [34]. It causes a prolonged (up to two years) and profound depletion of all these cell types. It is used off-label as an alternative induction antibody, where it proved to be at least as efficient as other induction antibodies (anti-IL2 or rATG) at preventing rejection in predominantly short-term studies spanning the first year after transplantation [35–40]. In several of these trials, it even enabled lower doses of maintenance immunosuppression. Its ease of administration (one or two doses of 30 mg subcutaneously or via a peripheral venous line) and lower incidence of infusion reactions are important benefits in clinical practice compared to the more complicated administration of rATG. Its efficacy and even superiority in some trials are less well established beyond the first year of transplantation.

The profound and prolonged lymphopenia raises concerns about an increased risk of infections and malignancy. In the predominantly short-term data that are available, this was not observed [41]. More recently, long-term retrospective

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analyses do point out a possible increase in patient mortality, infection-related mortality [42, 43], and even allograft loss [44]. The latter observation could possibly be explained by a tendency to use lower maintenance immunosuppression in patients treated with alemtuzumab. Prospective long-term data are urgently needed to confirm these observations.

10.2.2.4 Basiliximab

Basiliximab is a chimeric monoclonal antibody that consists of human heavy and light chain constant regions and murine heavy and light chain variable regions that specifically bind the α chain (also known as CD25) of the interleukin-2 (IL-2) receptor, which is upregulated on activated T cells. By binding CD25, basiliximab blocks the IL-2-mediated activation and proliferation of T cells, an essential step in the allo-immune response (signal 3 of the T-cell activation model).

Basiliximab on its own is a mild immunosuppressant and is only used to prevent rejection during induction where it is preferred over ATG in the absence of a high immunological risk. Because basiliximab has a prolonged half-life, it is administered in a limited dosing regimen. Typically, two doses of 20 mg, one preoperatively and one on day 4 after transplantation, are sufficient to saturate the IL-2 receptor α chain for about 30–45 days. Basiliximab is generally well-tolerated and only rarely leads to first-dose reactions due to its predominantly human structure.

Another humanized anti-CD25 antibody called daclizumab was once developed for the same indication but is currently no longer available.

10.2.2.5 Rituximab

Rituximab is a chimeric (human IgG1 constant regions and murine variable regions) monoclonal antibody that targets the CD20 antigen on the surface of mature B lymphocytes and pre-B cells. This leads to a rapid depletion of circulating B cells through antibody- and complement-dependent cytotoxicity and B-cell apoptosis. Rituximab does not deplete other B-cell populations such as stem cells, pro-B cells, terminally differentiated plasma cells, and plasmablasts, as these cells do not express CD20 on their surface.

In the field of transplantation, it is used to reduce high levels of preformed anti-HLA or anti-ABO antibodies during desensitization regimens prior to HLA- or ABO-incompatible transplantation. The standard dosage is 375 mg/m^2. Infusion reactions are quite common and similar to those of ATG (fever, chills, and rigors). To mitigate these reactions, premedication with corticosteroids, antihistamines, and acetaminophen is recommended. A profound and prolonged B-cell depletion is seen after infusion (usually 6–12 months).

10.2.2.6 Intravenous Immunoglobulins

Intravenous immunoglobulin (IVIG) is a preparation made from pooled plasma of healthy blood donors and is unselected, containing a wide variety of mainly IgG molecules with a distribution similar to normal serum. Selected preparations also exist, for example, CMV hyperimmune globulin from blood donors with high anti-CMV antibody titers. IVIG has been used in transplantation as part of

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desensitization therapy prior to ABO- or HLA-incompatible transplantation and for the treatment of antibody-mediated rejection (AMR). IVIG is considered to have immunomodulatory properties through several mechanisms, among which are [1] Fcγ receptor blockage, [2] accelerated degradation of pathogenic antibodies through FcRn saturation, [3] neutralizing anti-idiotype antibodies, [4] inducing B-cell hyporesponsiveness through interaction with the B-cell receptor (BCR) and alterations in downstream signaling, and [5] increased supply of regulatory T-cell (T_{reg}) epitopes leading to inhibition of the T- and B-cell-mediated immune response [45].

The clinical evidence for the use of IVIGs in preventing or treating AMR is limited to single-center experiences with often historical controls as reference and as part of a broader desensitization or anti-rejection therapeutic regimen. The only randomized study available is the IG02 study, which was a multicenter RCT of IVIG versus placebo in 98 highly sensitized patients awaiting kidney transplantation. IVIG was proven superior to placebo in reducing anti-HLA antibody titer ($p = 0.004$) and improved transplantation rate (31% versus 12%, one-tailed $p = 0.0137$) [46].

Standard dosing is 1–2 g/kg with slow infusion rates to prevent infusion reactions and volume overload. Infusion reactions are usually mild and mostly resolve after temporarily discontinuing the drug or reducing the infusion rate. Rare complications include acute tubular injury due to osmotic nephrosis secondary to sucrose or sorbitol present in some IVIG preparations, aseptic meningitis, and thrombotic complications.

10.2.2.7 Belatacept

Belatacept selectively inhibits T-cell activation by acting as a co-stimulatory blocker. It consists of a human fusion protein combining the Fc region of human IgG1 with cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4). By binding to CD80 and CD86 on antigen-presenting cells and preventing the binding of CD28, belatacept disrupts signal 2 required for T-cell activation (see Figure 10.1).

Belatacept is available exclusively as an intravenous formulation. It was developed in kidney transplantation, where it is currently FDA-approved as an alternative to CNIs in maintenance immunosuppressive therapy alongside MPA and prednisolone. In daily practice, its use is commonly reserved for patients who cannot take CNIs due to toxicity or problems of adherence. When comparing belatacept to CNIs as initial maintenance immunosuppression in kidney transplant recipients, belatacept has similar results on patient and graft survival rate. Switching from a CNI-based regimen to belatacept could possibly lead to better long-term allograft function and an improved metabolic profile due to its non-nephrotoxic and non-diabetogenic nature, which would translate into reduced long-term risk of death and graft loss [47]. However, belatacept appears to be less effective at preventing acute rejection. Furthermore, belatacept is associated with an increased risk of post-transplant lymphoproliferative disease (PTLD), particularly in patients who are seronegative for Epstein-Barr virus (EBV) and progressive multifocal leukoencephalopathy (PML). Therefore, it should not be given to EBV-seronegative patients or those with unknown EBV status. Additionally, observational studies

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have reported higher rates of opportunistic infections, such as cytomegalovirus (CMV) and Pneumocystis pneumonia [48].

Following the early promising results in kidney transplant recipients, trials in other types of solid organ transplantation followed. In lung transplant recipients, one pilot RCT showed increased mortality without improvement in acute or chronic rejection rates with belatacept compared to a typical transplant regiment [49]. In a phase II trial where liver transplant recipients were primarily treated with belatacept, improved renal function was observed, but they did have increased rates of acute rejection, graft loss, and death, leading to early termination of the study [50]. Nevertheless, there might still be a role for belatacept for posttransplant conversion in patients with CNI-associated renal dysfunction. In heart transplant patients, the combination of belatacept with a reduced CNI dose resulted in stabilization of renal function, but this did not reach statistical significance [51]. Another way to mitigate the increased risk of rejection observed with belatacept could be a very slow taper of CNIs [52]. Larger, prospective future studies are needed to further evaluate the efficacy, safety, and role of belatacept.

10.2.3 Typical Regimens in Maintenance Immunosuppression

Maintenance immunosuppression has the goal of preventing allo-sensitization and rejection while minimizing the risks associated with long-term immunosuppression, most importantly opportunistic infections and malignancy. It is tailored to every individual patient based on the perceived risks of rejection and toxicity (Table 10.1).

Induction immunosuppression is the more intense regimen used to prevent acute rejection in the immediate peri- and postoperative period. It usually consists of high-dose corticosteroids and a depleting or non-depleting antibody on top of maintenance immunosuppression. The choice between a depleting antibody (ATG) and a non-depleting antibody (basiliximab) is based on the perceived risk of rejection. ATG has been shown to be superior in high-risk transplantation. More aggressive induction is performed in the case of ABO- or HLA-incompatible transplantation,

Table 10.1 Risk factors for rejection [53, 54].

HLA sensitization (quantified as a high panel reactive antibody percentage) due to previous transfusion, pregnancy, or previous transplantation
Presence of donor-specific antibodies
Retransplant and previous rejection
Younger recipient age
Older donor age
Worse HLA matching
Delayed graft function
Deceased donor grafts (versus grafts issued from living donors in the case of kidney transplantation)
Cold ischemia time > 24 hours

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where transplantation needs to occur despite circulating donor-specific anti-ABO or anti-HLA antibodies. In this setting, desensitization is performed with the administration of rituximab 375 mg/m^2 one month before transplantation, extracorporeal removal of circulating antibodies with plasmapheresis or immunoadsorption (vide infra), and perfusion of IVIG.

Maintenance therapy classically consists of a "triple-drug regimen" made up of a CNI, an antiproliferative agent, and low-dose corticosteroids. Tacrolimus is often favored over cyclosporine due to its higher potency. As an antiproliferative agent, MPA is the drug of choice due to its higher potency than azathioprine. Azathioprine is preferred in case of pregnancy given the important teratogenicity of MPA. mTOR inhibitors are increasingly used to reduce CNI exposure in patients with an increased risk of post-transplant malignancy.

Over recent years, this classical triple therapy regimen has been downgraded to corticoid-free regimens whenever possible to prevent the vast array of side effects related to long-term treatment with corticosteroids. In that case, dual immunosuppressive therapy consisting of a CNI and MPA or mTORI is prescribed. Attempts to avoid CNIs and their nephrotoxicity have also been performed. CNI-free therapy combining mTORI, MPA, and corticosteroids is associated with an increased risk of rejection and is not standard practice. Belatacept in conjunction with MPA and corticosteroids can be used in patients with evidence of CNI toxicity.

10.3 Treatment of Rejection

In all types of solid organ transplantation, histological criteria are developed to distinguish antibody-mediated (AMR) from T-cell-mediated rejection (TCMR). In kidney and liver transplantation, this is done based on the Banff criteria [55, 56], whereas in heart and lung transplantation, the ISHLT (International Society of Heart and Lung Transplantation) guidelines are available [57, 58]. Therapy is tailored to this histopathological diagnosis. Sometimes combined AMR and TCMR is observed. In this case, a combination of both strategies is often performed. High-quality data (from randomized controlled trials (RCTs) or large observational studies) are rare and are largely produced in the field of kidney transplantation. Rejection treatment in other solid-organ transplants (SOT), such as heart, lung, and liver transplantation, is mainly derived from data in kidney transplantation and is therefore highly similar. Besides the initial aggressive treatment regimens for AMR and TCMR, maintenance immunosuppression needs to be intensified, and dual immunosuppressive regimens will be switched to standard "triple therapy."

10.3.1 Treatment of Antibody-Mediated Rejection

Treatment for antibody-mediated rejection (AMR) is aimed at the removal of circulating donor-specific antibodies (DSAs) and inhibition of their production. As high-quality data are rare, no definite standard of care exists, and specific modalities differ from center to center. Most often the treatment of AMR consists

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of the combination of high-dose steroids, plasmapheresis (or immunoadsorption in the case of ABO incompatible kidney transplantation), and IVIG for all types of SOT [59].

10.3.1.1 Extracorporeal Removal of Circulating IgG: Plasmapheresis and Immunoadsorption

The term apheresis refers to the general methodology of removal of one or more components from the blood. Plasmapheresis or therapeutic plasma exchange (PLEX) is a non-selective apheresis technique in which plasma is separated from whole blood and then discarded. The removed plasma volume needs to be replaced by colloid solutions, most commonly human albumin solutions (4–5%) or fresh frozen plasma (FFP). As PLEX unselectively removes all plasma macromolecules, it leads to a progressive bleeding tendency due to loss of coagulation factors. In case of an increased bleeding risk, FFP is used instead of albumin or together with albumin where FFP is used at the end of every PLEX session. Double filtration plasmapheresis (DFPP) adds a filtration step in cascade with plasma separation to selectively remove larger molecules such as immunoglobulins. Smaller plasma proteins and albumin are reinfused to the patient. This avoids the need for large-volume substitution. An even more selective technique is immunoadsorption (IA), in which the separated plasma is guided over adsorption columns coated with a specific antigen. This can be semi-selective, such as in the removal of circulating IgG using IA columns coated with staphylococcal protein A that selectively binds IgG through high-avidity interaction with their Fc portions. In semi-selective IA, the columns can also be reused, which increases its cost-efficiency. Selective IA is used to selectively remove circulating immunoglobulins reacting with a specific antigen. For example, columns coated with blood group antigens A or B are used during desensitization before ABO-incompatible transplantation (see Figure 10.5).

As plasmapheresis removes DSAs from the vascular compartment, it is used during desensitization before HLA-incompatible transplantation and for the treatment of AMR. It can also be used for desensitization before ABO-incompatible transplantation, although increasingly, selective anti-A or B immunoadsorption is used for this indication [60].

Data supporting the use of PLEX are limited with hardly any controlled studies available and high variability between studies. A well-designed RCT will probably never be performed as PLEX has become the current standard-of-care [59, 61]. One older, positive RCT in 44 patients with acute kidney graft rejection unresponsive to steroids with anti-HLA antibodies and predominant vascular lesions on kidney biopsy reported a significant beneficial effect of PLEX with a reported graft loss in 7/23 (30%) in the PLEX group compared with 17/21 (81%) in the control group [62]. Other studies failed to confirm this beneficial effect or reported a much smaller difference in graft survival [63].

The role of IA in desensitization before ABOi transplantation is well established [60]. Its use in desensitization before HLA-incompatible transplantation is more experimental as is its use in the treatment of AMR, although data seem promising

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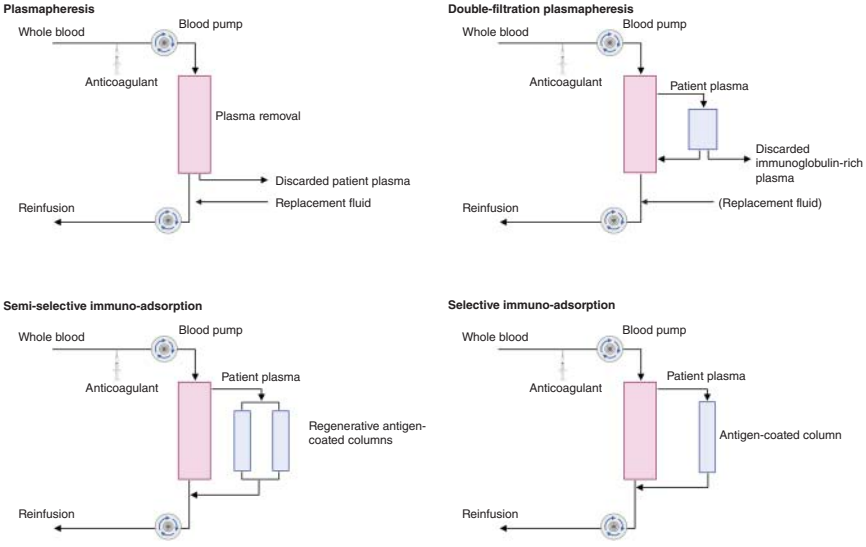

Figure 10.5 Different techniques of extracorporeal removal. Plasmapheresis entails the non-specific removal of patient plasma, after which it is replaced by colloid solutions such as human albumin 5% or fresh frozen plasma. Double-filtration plasmapheresis makes use of an additional size-selective filter isolating larger plasma proteins such as immunoglobulin. Semi-selective immunoadsorption uses two regenerative columns, which are coated with a semi-selective antigen such as protein A. The two columns are used in alternance: when the first column is saturated, plasma is sent over the second column while the first is being regenerated. Selective immunoadsorption is currently only performed for blood group incompatible transplantation, where the single-use columns are coated with blood group A or B saccharides. Created in BioRender.com.

in kidney transplantation [64–66]. In other types of SOT, too little evidence exists to recommend IA to prevent or treat AMR. As such, only PLEX is recommended.

10.3.1.2 Rituximab

Rituximab is a monoclonal antibody directed against CD20, a surface marker for B cells. Its mechanism of action is mentioned earlier in this chapter. Different small series and retrospective studies have suggested a benefit from the addition of rituximab in the treatment of AMR. The only prospective RCT in kidney transplantation studied the effect of rituximab as an add-on treatment to plasmapheresis and IVIG in 38 patients with AMR. No significant benefit was observed [67]. Experience with the second-generation anti-CD20 monoclonal antibody obinutuzumab is even more limited and not more convincing. In non-kidney solid-organ transplant recipients, rituximab is sometimes used for the treatment of AMR, although no prospective or high-quality data exist [68–70].

10.3.1.3 Anti-CD38

Daratumumab is an IgG1 human monoclonal antibody targeting CD38+ plasma cells. It is part of the first-line approach for patients with multiple myeloma. The immunoglobulin-secreting plasma cells are a logical target in AMR, and thus

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daratumumab qualifies for the treatment of AMR along the same lines as proteasome inhibitors, with daratumumab having the advantage of a more manageable toxicity profile. Daratumumab has been shown to significantly reduce DSA levels in a primate model of AMR [71] and in several case reports of highly sensitized patients awaiting kidney transplantation [71, 72]. This has also been observed in both heart and kidney transplant patients with refractory AMR in whom daratumumab led to successful treatment of AMR [73, 74]. In several of these reports; however, a rebound increase of DSAs was observed several months after treatment, sometimes leading to the reappearance of AMR necessitating re-treatment with daratumumab.

Felzartamab, a newer CD38-targeting monoclonal antibody, was recently tested in a small RCT in 22 kidney transplant patients with late AMR. At week 24, resolution of active AMR occurred in 9/11 (82%) patients in the felzartamab group versus 2/10 (20%) in the placebo group. No important side effects were noted. Just as for daratumumab, reappearance of AMR was observed in some patients one year after treatment. Besides the DSA-lowering effect, felzartamab was believed to treat AMR through its effect on NK cells, which are also CD38-positive [75].

Similar data were obtained with isatuximab, another CD38-blocking antibody. Twenty-three highly sensitized patients were treated in a pre-kidney transplantation desensitization setting where isatuximab was associated with a decrease in anti-HLA antibodies and increased access to kidney transplantation [76].

10.3.1.4 Proteasome Inhibitors

Proteasome inhibitors (PIs) are standard-of-care drugs in the treatment for plasma cell neoplasms. They inhibit the proteolytic activity of the proteasome, leading to intracellular protein accumulation, even aggregation, and the activation of apoptotic pathways and cell death. As plasma cells produce high amounts of immunoglobulin, they are highly dependent on a functioning proteasome and especially susceptible to PIs. There is an obvious rationale in AMR in an attempt to decrease DSA production. Small uncontrolled case reports where PIs were used on top of standard AMR therapy described potentially beneficial effects. The only placebo-controlled study, however, failed to show benefit [77]. Furthermore, proteasome inhibitors have substantial gastrointestinal, hematologic, and neurologic toxicity.

10.3.1.5 Anti-IL-6

Interleukin 6 (IL-6) is a multifunctional cytokine involved in the development, maturation, and activation of T cells, B cells, and plasma cells. Interleukin 6 blocking agents are approved for the treatment of multiple autoimmune diseases. Tocilizumab is a humanized monoclonal antibody directed at the interleukin-6 receptor (IL-6R). Siltuximab and clazakizumab are agents directed against IL-6 itself. As IL-6 is a key cytokine in plasma cell activation and was shown to be increased in patients with AMR, IL-6 blockade could also be of interest in the treatment of AMR [78]. After a mouse model of allosensitization showed that anti-IL-6R treatment resulted in an attenuation of the alloantibody recall response with lower DSAs and a shift from Th_{17} cells and T_{fh} cells toward an increase in T_{reg} and B_{reg} cells, 11 kidney transplant patients with resistant DSA+ AMR were

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offered rescue treatment with tocilizumab [79]. This resulted in a stabilized kidney function in 10/11 and a decrease in DSA in 6/11 [80]. Several non-controlled studies followed, evaluating tocilizumab and clazakizumab for the treatment of refractory AMR, chronic antibody-mediated rejection (cAMR), and as part of desensitization before kidney transplantation. These studies confirmed the observed decrease in DSAs and increase in T_{reg} cells and showed promising clinical results [81–83]. No randomized controlled data are available for the evaluation of tocilizumab in kidney transplant rejection, neither in AMR nor cAMR. The placebo-controlled phase III trial IMAGINE evaluating clazakizumab in kidney transplant patients with cAMR was terminated early after a first interim analysis showed that the primary efficacy outcome was unlikely to be met [84]. No difference in the evolution of kidney function was observed between the two groups. This is somewhat disappointing after the suggested beneficial effect in earlier non-controlled case series. It might be the case that these agents only have an added value when used as part of a multitargeted approach. Further research is needed to better define their potential role in the field of transplantation.

10.3.1.6 Complement-Directed Therapy: Eculizumab

Antibody-mediated cytotoxicity is in part mediated through activation of the classical complement pathway. This is also illustrated by the observed endothelial C4d deposits in AMR biopsies. Complement-activating DSAs also infer a worse prognosis than non-complement-activating DSAs [85]. This makes the complement cascade an interesting target to prevent or treat humoral alloreactivity [86]. Different complement inhibitors have been used off-label in the treatment of AMR.

Eculizumab, a monoclonal antibody targeting C5, provides distal inhibition of the complement cascade and prevents formation of the membrane attack complex. After several small observational studies reported largely promising though somewhat conflicting results of eculizumab in the prevention [87–90] and treatment of AMR [91–94], a major international effort was undertaken to better clarify the role of eculizumab in preventing AMR [85]. In this study, 116 DSA^+ kidney transplant recipients were prospectively screened for complement-activating (i.e. C1q binding) DSAs and either received standard of care (plasmapheresis and IVIG) or eculizumab. They observed important differences in histology, DNA expression profiles, and response to eculizumab between the two groups with a three-month rejection incidence rate of 56% (SOC) versus 19% (eculizumab) ($p = 0.001$) in patients with complement-activating DSAs versus no effect of eculizumab in patients with non-complement-activating DSAs (9% versus 13%, $p = 0.65$). It thus seems that eculizumab is of particular interest in preventing early AMR in patients with DSAs that activate the classical complement cascade [85, 95]. Only anecdotal evidence exists in non-kidney SOT, making it impossible to make any firm conclusions about the role of eculizumab in these patients [96–98].

Over recent years, a gain of interest was observed for proximal inhibition of the classical complement cascade as this would prevent activation of all upstream complement factors to C5 where eculizumab has its effect. Several preclinical and observational studies of either isolated human C1 inhibitor (C1INH) or

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recombinant human C1 inhibitor (rhC1INH) showed promising results [99–102]. C1INH or C1 esterase inhibitor is a physiologically present protease inhibitor that inactivates the classical and lectin pathways through interaction with C1r, C1s, FXIIa, FXIa, and kallikrein. In doing so, it prevents spontaneous complement activation. Despite these initial results, two placebo-controlled phase 3 studies with C1INH in AMR were prematurely terminated because of futility (NCT03221842 and NCT02547220).

10.3.1.7 Imlifidase

Imlifidase or IdeS (IgG-degrading enzyme derived from Streptococcus pyogenes) is one of the most recently developed drugs in the field of AMR. It is an endopeptidase derived from *Streptococcus pyogenes*, which cleaves human IgG at the hinge region and was shown to rapidly reduce circulating DSA levels [103]. Imlifidase was shown to make kidney transplantation possible for highly immunized patients by rendering the FCXM (flow cytometric crossmatch) negative within 24 hours in almost 90% of patients. Its use is hampered by the reappearance of IgG and a rebound increase in DSAs within 1–2 weeks after transplantation with an observed AMR incidence of 38% [104]. These results have led to the conditional approval by the European Medicines Agency (EMA) of Imlifidase for desensitization of highly sensitized recipients with a positive crossmatch against an available deceased donor. Its role in the treatment of AMR is less clear with a recent multicenter RCT reporting a more rapid decrease in DSAs with imlifidase compared to plasmapheresis but no benefit in terms of allograft survival and kidney function. There was even a trend toward more allograft loss in the imlifidase arm [105].

10.3.2 T-Cell-Directed Therapy

The treatment of TCMR has changed little over time and does not differ substantially between different types of SOT. It usually consists of pulse corticosteroid therapy (usually IV methylprednisolone 250–500 mg per day during three consecutive days), which is either followed by an immediate return to maintenance dose or a slow taper. Most TCMR lesions respond rapidly to corticosteroids. In case of more severe lesions or insufficient response to corticosteroids, rATG is added (1.5–3 mg/kg per day during 3–7 days with a total dose of 3–10 mg/kg). There are very few data supporting a certain dose or duration of either glucocorticoids or rATG in all types of SOT, and treatment protocols are known to differ slightly between transplant centers [106, 107]. Maintenance immunosuppression is also intensified whenever possible. This is sometimes the only action performed in cases of very mild or subclinical TCMR.

Sometimes alemtuzumab is used instead of rATG (in case of fear of anaphylactic reactions, for example) or as salvage therapy for patients with refractory rejection. Indeed, alemtuzumab appears to be effective in severe treatment-resistant rejection [108]. Recent retrospective data, however, show an increased risk of death (hazard ratio 1.75, 95% CI 1.28–2.39) and death due to infection (hazard ratio 2.36, 95% CI 1.35–4.11) [43].

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A repeat biopsy is usually only performed in case of unsatisfactory clinical response to treatment, although evidence exists that this could be useful to assess histological remission in every patient with TCMR, as subclinical lesions can persist [109].

10.4 Conclusions

The prevention and treatment of AMR and TCMR are often based on very limited data. High-quality RCTs are rare in the field of transplantation and even more rare when it comes to the treatment of rejection. Furthermore, definitions of AMR and TCMR have evolved over time; maintenance drug regimens have changed from cyclosporin/AZT to CNI/MPA, and clinical endpoints defining treatment success or failure vary tremendously between studies. This makes it difficult to make hard conclusions regarding the best prevention and treatment of different subtypes of rejection. This has also led to a marked heterogeneity in clinical practice between transplant centers. Increasing efforts are being undertaken to standardize clinical outcome parameters to facilitate further high-quality research in this field [110]. Furthermore, the current diagnosis of rejection is still primarily based on histopathology. In the future, this will likely be supplemented or even replaced by molecular analyses, leading to a better understanding and classification of different types of rejection, paving the way for more individualized precision medicine.

Several promising drugs are also being tested in the preclinical or early clinical phases. Combined inhibition of BCMA+ plasma cells and $CD3^+$ T cells using the bi-specific anti-BCMA-CD3 monoclonal antibody is being tested for desensitization in kidney transplantation (NCT05092347). FcRn inhibitors block the neonatal Fc receptor responsible for IgG recycling, which shortens the half-life of IgG and has been shown to reduce DSA levels [111]. In this light, Efgartigimod is currently being tested for the treatment of AMR in kidney transplant recipients (NCT06503731). T_{reg} cell therapy, either as endogenous T_{reg} cells or as chimeric antigen receptor-T_{reg} cells (CAR-T_{reg} cells), shows very exciting results [112, 113] and is currently being tested in phase I–II studies (NCT04817774 and NCT05234190).

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11

Stemcell Transplantation

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11.1 State-of-the-Art of Allogeneic Hematopoietic Cell Transplantation

Since E Donnall Thomas performed the first bone marrow transplant in 1957 [1], landmark advances have increased the safety, efficacy, and availability of transplantation and have enabled curative therapy for over one million patients suffering from life-threatening malignant and nonmalignant blood disorders [2]. Increased safety and efficacy of HCT have come about through improvements in the transplant procedure to lower transplant-associated complications of graft rejection and graft-versus-host disease (GVHD), and the immunobiology underlying leukemia relapse (Table 11.1). Increased availability has been possible through a more complete understanding of the HLA barrier and the limits of donor disparity. Basic and clinical research findings have led to the increased use of donors other than genotypically-matched siblings, which has greatly increased the availability of HCT for patients who lack matched family member donors.

Worldwide, the diseases for which HCT can provide a cure or produce prolonged remissions have increased [3, 4]. In 1990, the European Group for Blood and Marrow Transplantation (EBMT) began to survey the use of HCT by centers in Europe [5], providing first-hand knowledge of the application of HCT for specific blood disorders and the kinds of donor stem cell sources and transplant regimens used. The survey has been an invaluable chronicle of how the practice of HCT continues to expand and diversify. The 2021 EBMT survey showed that 19,798 allogeneic transplants were performed in Europe in 2019 for patients with myeloid (25%) and lymphoid (64%) malignancies and nonmalignant disorders (7%). Sentinel developments include the increased use of unrelated volunteer donors and haploidentical related donors, the latter of which showed an 11% increase from 2018 to 2019 alone. Notably, the use of peripheral blood stem cells has increased over bone marrow for haploidentical transplantations. The annual surveys continue to inform the practice of HCT.

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Table 11.1 Glossary of transplant terms.

Term	Description
Patient comorbidity	Risks imparted by the patient's pretransplant functional (fitness) status.
Conditioning regimen	Chemotherapy with or without radiation that is administered immediately prior to the infusion of donor stem cells on transplant day 0.
Myeloablative Conditioning (MAC)	High-dose chemotherapy with or without radiation that leads to ablation of host stem cells and requires a rescue of stem cells to restore hematopoiesis.
Reduced-Intensity Conditioning (RIC)/Non-Myeloablative Conditioning (NMA)	Lower-intensity regimens of chemotherapy with or without radiation that are myelosuppressive and rely on graft-versus-leukemia effects to eradicate underlying disease.
GVHD prophylaxis	Regimens aimed at lowering the risk of severe GVHD. Regimens include pharmacologic agents given in combinations that suppress donor (graft) recognition of the host or to remove of T cells (ex vivo or in vivo).
T-replete graft	A stem cell graft which is not manipulated and contains donor T cells.
T-cell deplete graft	A stem cell graft that has had donor T cells removed before infusion, or, use of pharmacologic agents in vivo to deplete T cells after infusion.
Graft-versus-leukemia effect	Donor recognition of determinants expressed by the patient's leukemia, against which a cytotoxic response leads to lower relapse.

In 2015, the American Society for Blood and Marrow Transplantation (ASBMT), formally renamed the American Society for Transplantation and Cellular Therapy (ASTCT), evaluated the evidence for the role of HCT in the treatment of blood malignancies at specific stages of the disease [6]. The ASTCT has updated the survey [4] and highlighted the diseases and stages for which HCT is considered the standard of care, which are acute myeloid leukemia (AML) in complete remission (CR) 1, intermediate risk; AML CR1 high risk; AML CR2; AML CR3 or beyond; AML in relapse; acute lymphoblastic leukemia (ALL) in CR1, standard risk; ALL CR1 high risk; ALL CR2; ALL CR3 or beyond; myelodysplastic syndrome (MDS) intermediate-2/high risk; therapy-related AML/MDS in CR1. These "indications" guide the timing of HCT when the benefits of HCT for disease control outweigh the risks of the procedure.

Data from a single transplant center has illustrated the substantial reductions in transplant-related complications and improved overall survival of patients over time, attributable to many factors including improvements in the transplant procedure itself, supportive care, and approaches for the selection of the optimal donors [7]. The lowered transplant-related complications of organ toxicity, cytomegalovirus (CMV) viremia, and acute and chronic GVHD were observed

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in patients receiving more intensive (myeloablative conditioning, MAC) as well as less-intensive conditioning regimens (reduced-intensity conditioning [RIC]; non-myeloablative [NMA]) and allografts from related and unrelated donors. This collective experience illustrates the many factors that influence the success of a transplant. An appreciation for the fitness of a patient to safely undergo HCT has been an important area of clinical research and has played a key role in the planning of HCT for a given patient and in the broadening of HCT to patients at more advanced ages.

11.1.1 Patient Eligibility

The optimal timing of HCT for a patient is influenced by the patient's overall health fitness and the disease stage and urgency of the transplant for the control of the underlying disease. The combination of functional assessment and disease-specific parameters provides the basis for patient eligibility and timing of transplantation. Over the long history of HCT, the fitness of a patient has been described with different metrics to estimate the potential for life-threatening organ toxicity and complications from the transplant procedure itself. Patient eligibility for transplantation takes into consideration disease factors as well as the overall functional status of the patient. Assessment prior to HCT defines the risks and benefits of HCT to lower morbidity and mortality due to transplant-related complications. The timing of transplantation for any given patient is influenced by patient-specific factors and is optimally performed when the benefits of HCT greatly outweigh the risks of the procedure itself. A number of robust tools are available to assist clinicians in the assessment of patient functional status. One of the oldest tools is the Karnofsky Performance Scale Index developed in 1948, which continues to provide essential information on the fitness of a patient [8]. The Eastern Cooperative Cancer Group (ECOG) [9] and EBMT [10] have each developed robust measurements of patient performance status. More recently, the Comorbidity Index (CI) [11] is available as a web-based application which considers organ-specific history and laboratory measurements such as left-ventricular cardiac ejection fraction, pulmonary function tests, renal and hepatic function, and psychosocial health and well-being. Based on these parameters, an overall score is calculated. The CI score has been validated to be prognostic for non-relapse mortality and overall mortality after transplantation. These tools are used to supplement the in-depth analysis of the patient's underlying malignancy.

The lowering of non-relapse mortality for older patients with reduced-intensity and non-myeloablative transplant conditioning regimens has enabled older patients to safely undergo allogeneic HCT [12–16]. As the average age of adult patients increases, the ages of their full siblings also increase; similar to screening patients for their functional status, candidate-related donors for transplantation should be evaluated early in the process of transplant planning. Family members who are available and interested in serving as a donor may be screened for their health history and family members whose health screen suggests suitability may undergo HLA typing.

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11.2 Haploidentical HCT

Although HLA-genotypically-matched siblings have been considered the preferred stem cell source, superior clinical outcomes after haploidentical related donor HCT have redefined the donor landscape for patients who lack matched siblings [17–23]. Patients of diverse racial and ethnic backgrounds have a disproportionately lower probability of identifying HLA-matched unrelated donors and have benefited from haploidentical related donor HCT [24] as have patients whose high-risk disease status requires urgent transplantation [25].

The formal definition of a haploidentical related donor is a family member who shares one complete chr 6 with the patient and who has inherited a different chr 6 for the non-shared haplotype (Figure 11.1). Typing for six HLA loci, HLA-A, -B, -C, -DRB1, DQB1, and -DPB1, provides the basis for assigning haplotypes within a family, and often the supra-typic class II loci (HLA-DRB3, -DRB4, and -DRB5), HLA-DQA1, and HLA-DPA1 are considered in the assessment of haplotypes within a family. In cases where there is fortuitous sharing of HLA alleles between parents, comprehensive typing of children and family members will increase the probability of identifying a true haploidentical related donor. The non-shared haplotypes between haploidentical relatives may differ for one or more alleles.

The HLA complex is inherited through classic Mendelian segregation, and hence, the probability that a full sibling is haploidentical is 50%. Among patients referred for haploidentical related donor HCT at one center, over 96% of patients had a haploidentical donor and averaged 2.5 donors per patient [26]. The likelihood of identifying second degree haploidentical relatives has similarly been investigated [27].

11.2.1 Historical Development of Haploidentical Related Donor Transplant Regimens

There was early recognition of a dual need to facilitate engraftment while, at the same time, reducing life-threatening GVHD. In the 1980s, the experience with unmanipulated, T-replete grafts demonstrated high rates of graft rejection and GVHD [28]. Although methods to remove T cells from the graft were highly successful in lowering severe acute GVHD, graft rejection was a limitation [29, 30]. Graft rejection was overcome through the use of large doses of T-cell depleted grafts which yielded full donor engraftment and low regimen-related toxicity [31]. This early experience paved the way for the development of different methodologies for the removal of T cells including *ex vivo* T-cell depletion using CD3/CD19 depletion and anti-CD52 with alemtuzumab [32], adoptive transfer of T-regulatory cells [33], and αβ T-cell/B-cell depletion [34–36].

This rich history has led to four approaches for haploidentical-related donor HCT today: T-replete grafts with posttransplant cyclophosphamide (PTCy) [17, 37, 38], T-replete grafts with G-CSF-primed bone marrow and anti-thymocyte globulin (ATG) [39–42], T-cell depleted grafts followed by naïve cell-enriched T cells [43, 44], and selective αβ T-cell and B-cell depletion [45–48]. The extensive preclinical and clinical studies that have led to modern-day approaches for haploidentical

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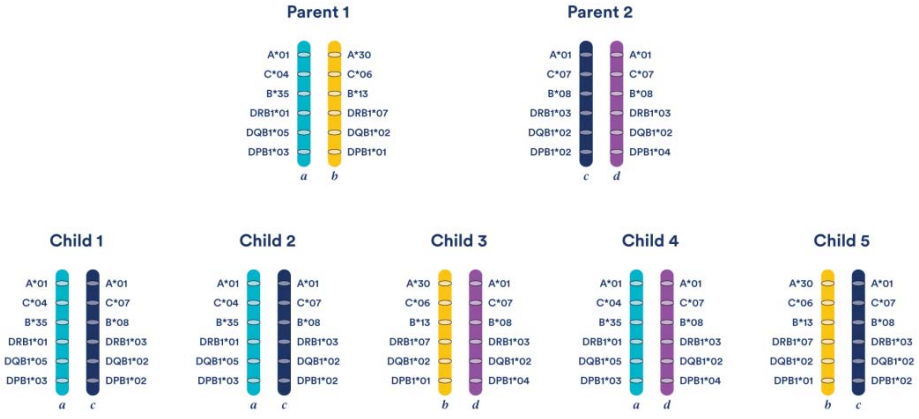

Figure 11.1 A Family Study. Six-locus HLA-A, -C, -B, -DRB1, -DQB1, and -DPB1 typing is performed for parents and five children. Haplotype segregation demonstrates three concepts. Parental haplotypes "c" and "d" differ only at HLA-DPB1. Six-locus typing is needed to discriminate two different haplotypes. Child #1 and Child #2 are identical-by-descent and are "HLA genotypically-matched siblings" to each other. Child #3 and Child #5 are haploidentical to each other due to two different A1-C7-B8-DR3-DQ2 haplotypes (haplotypes "c" and "d"). Their haploidentity can be confirmed by typing HLA-DPB1.

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related donor transplantation are beyond the scope of this chapter, and the reader is invited to outstanding reviews on this subject [44, 49–53]. A brief overview of T-replete haploidentical related donor transplantation using PTCy is presented below as the emerging associations between HLA gene features and outcome have been derived from large cohorts receiving this modality.

11.2.2 Haploidentical Related Donor HCT with Posttransplant Cyclophosphamide (PTCy)

The wide availability of cyclophosphamide, the ease of its administration, the relatively low side effects experienced by patients, and no manipulation of the graft have all contributed to the exponential use of PTCy-based regimens for haploidentical related donor HCT [3, 17, 18, 21, 54]. Early preclinical studies showed that PTCy can facilitate stable engraftment and chimerism without severe GVHD in mismatched murine models [55]. Early clinical trials employed a single dose of PTCy in haploidentical bone marrow transplantation using non-myeloablative conditioning [37]. Subsequent studies explored a two-dose regimen which lowered the risks of chronic GVHD after haploidentical marrow transplantation using non-myeloablative conditioning. Current widely used regimens administer PTCy on two successive days (days +3 and +4) after the day 0 infusion of stem cells [17].

PTCy exerts its immunosuppressive effects in three ways. The $CD4^+$ alloreactive effector cells infused with a T-replete allograft have high sensitivity in the immediate post-infusion period. Administration of PTCy during this critical period selectively reduces $CD4^+$ effector cell proliferation and induces a functional impairment of $CD4^+$ as well as $CD8^+$ effector T cells that survive the regimen, thus reducing both graft rejection and GVHD. PTCy furthermore facilitates the development of peripheral tolerance via regulatory T cells. PTCy is used in concert with standard immunosuppression.

Outcomes following PTCy-based haploidentical related donor HCT have been chronicled across multiple single-center and registry studies for both malignant and nonmalignant [56–58] diagnoses and for older adults [59]. Haploidentical related donor HCT compares favorably to HLA-matched sibling HCT [21, 22, 40, 60–64] and HLA-10/10-matched unrelated donor HCT [19–21, 23, 65–69], cord blood transplantation [70], or all three modalities [71]. For older adults, relapse and survival rates across age groups are similar with the use of reduced-intensity conditioning regimens and PTCy [59]. Nonmalignant blood disorders including sickle cell disease and severe aplastic anemia can be successfully treated with haploidentical HCT [57, 58, 72, 73].

A recent retrospective registry study demonstrated superior overall survival, GVHD-free/relapse-free survival, and chronic GVHD-free/relapse-free survival following haploidentical related donor HCT compared to cord blood, HLA-7/8 unrelated donor marrow, or HLA-7/8 unrelated donor peripheral blood stem cell transplantation particularly for patients receiving reduced-intensity regimens [74]. Two recent clinical trials are noteworthy. The Bone Marrow Transplant Clinical Trials Network (BMT CTN) #1101 trial randomized patients to haploidentical

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HCT with PTCy, tacrolimus, and mycophenolate mofetil (MMF) or cord blood transplantation with cyclosporine and MMF and observed a trend toward better overall survival at two years after HCT for patients undergoing haploidentical HCT. Most recently, BMT CTN #1703 compared two prophylaxis regimens, PTCy/tacrolimus/MMF and tacrolimus/methotrexate for patients receiving reduced-intensity conditioning and HLA-matched related donor, HLA-8/8-matched unrelated donor, or HLA-7/8-matched unrelated donor HCT. Patients who received a matched allograft and PTCy/tacrolimus/MMF had higher GVHD-free/relapse-free survival one year after transplantation compared to patients receiving tacrolimus/methotrexate [75].

In conclusion, clinical experience has shown high rates of engraftment with low regimen-related mortality and low GVHD following haploidentical related donor HCT. Furthermore, the beneficial reduction of severe GVHD in haploidentical HCT with PTCy can also be observed following HLA-matched transplantation [76–80]. The optimization of posttransplant immune reconstitution and prevention of disease recurrence after transplantation are at the forefront of future clinical studies.

11.3 Immunobiology of Haploidentical Related Donor Transplantation

The role of the HLA system in allogeneic HCT has been elucidated through a long history of clinical and basic research, with discoveries in HLA-mismatched HCT at the crux of those studies (Table 11.2). Whereas the significance of mismatching for individual loci has figured prominently in outcomes after unrelated donor and cord blood transplantation, new evidence on the effects of HLA mismatching has recently emerged for haploidentical related donor HCT, and many of those associations replicate the findings in unrelated donor HCT. This may in part reflect the need for larger, homogeneous populations of patients and haploidentical related donors who are typed at higher resolution to better understand the specific HLA factors that influence clinical outcome after haploidentical related donor HCT and who receive uniform transplant conditioning and GVHD prophylaxis; however, the exceedingly low rates of acute GVHD resulting from successful eradication or removal of alloreactive T cells, as described in the section above, also change the clinical endpoints that can be observed to have associations with HLA disparity. The dynamics of the transplant conditioning and GVHD prevention measures used today in haploidentical HCT shift the early posttransplant HLA-associated risk assessment more toward chronic GVHD and relapse.

The non-shared haplotype plays a central role in the immunobiology of HLA in haploidentical related donor HCT. However, evidence from HLA-mismatched unrelated donor HCT has highlighted the importance of matched alleles and haplotypes in clinical outcome. These germline-associated effects are also emerging as important sources of variation in haploidentical related donor HCT, and their consideration is essential to understanding the risks stemming from the mismatched allotypes contributed by the non-shared haplotype. New data on nonclassical class I and II

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Table 11.2 Glossary of genetic terms.

Term	Definition	Examples
Single nucleotide polymorphism (SNP)	The most simple form of human genetic variation in which the substitution involves two different nucleotides.	A/C; A/T; A/G.
Dimorphic/dimorphism	Two alternative nucleotide base substitutions at a given chromosomal marker that yield two alternative amino acid substitutions for the triplet codon.	HLA-B exon 1 position −21 rs1050458 C/T encodes Met versus Thr at the second position of the leader peptide. HLA-DRβ residue 86 Gly versus Val
Genotype	The combination of polymorphisms for a given marker or series of markers.	rs9277534 AG HLA-A1,2; B7,8; DR2,3
Haplotype	2 or more markers inherited in cis on the same chromosomal strand	MICA 129Met – STR A5.1 HLA-A1-C7–B8-DR3-DQ2
Identity-by-descent	Siblings who have inherited the same paternal and maternal HLA haplotypes.	Figure 11.1 child #1 and child #2
Haploidentical	Two related individuals who share one complete HLA haplotype and differ for the non-shared haplotype.	The degree of HLA identity between the non-shared haplotypes depends on the fortuitous sharing of markers on parental haplotypes (Figure 11.1 child #3 and child #5)
Graft-versus-host (GVH) vector of incompatibility	Presence of a polymorphism in the patient that is absent in the donor.	A basis for allorecognition that may lead to graft-versus-host disease. HLA-A1,2 patient and HLA-A1,1 donor.
Host-versus-graft (HVG) vector of incompatibility	Presence of a polymorphism in the donor that is absent in the patient.	A basis for allorecognition that may lead to graft rejection. HLA-A2,2 patient and HLA-A1,2 donor.
Bidirectional HLA mismatch	Presence of both the GVH and the HVG vectors of incompatibility.	HLA-A1,2 patient and HLA-A1,3 donor.
Leader peptide	The 9 amino acid protein product of exon 1 of HLA class I genes. Also known as the signal peptide.	HLA-B exon 1 leader peptide encodes Met or Thr at the second position.
Donor-specific antibodies (DSA)	Antibodies produced by the patient that are directed against mismatched HLA antigens in the donor.	A mechanism for graft rejection.
Copy neutral HLA loss (cnLOH)	One or more loci in class I and/or class II may be lost and replaced by a copy of the intact haplotype, resulting in no change in genomic copy number and acquired homozygosity. Also known as acquired somatic uniparental disomy (aUPD).	A mechanism of HLA loss.

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genes highlight the breadth of the immune response and the factors that modulate T- and NK-cell alloresponses in haploidentical related donor HCT.

Alloresponses in HCT are "bi-directional": host-versus-graft responses provoked by donor HLA allotypes that are not present in the patient and graft-versus-host recognition of patient determinants that are not present in the donor. The former is the basis for graft rejection, the latter for graft-versus-host disease and graft-versus-leukemia effects (GVLEs). The ability of the donor's immune system to mount an effective attack on residual patient leukemia cells is predicated on the presence of patient-mismatched HLA allotypes, without which no graft-versus-leukemia effect can be had. This is a critical concept in haploidentical related donor HCT, in which the loss of the non-shared mismatched haplotype leads to the loss of expression of the patient's mismatched HLA allotypes on leukemia cells, hence eliminating the very targets for successful eradication of the leukemia by the donor.

11.3.1 The Haplotype

The Third International Histocompatibility Workshop was held in 1967 in Turin, Italy. It was a pivotal workshop chaired by Ruggero Ceppellini, as it formally cemented the concept that HLA gene products are co-inherited as linked genes on the same chromosomal strand [81]. Ceppellini's landmark contribution is the basis for the definition of haplotypes between a patient and a haploidentical family member. Segregation of HLA follows classic Mendelian genetics (Figure 11.1). The inheritance of the same chr6 between the patient and haploidentical relative is known as the "shared haplotype". They differ for the "non-shared haplotype." Depending on fortuitous sharing of antigens between the parental haplotypes, the patient and haploidentical family member may be matched at discrete HLA loci of the non-shared haplotype.

In the family study shown in Figure 11.1, six-locus HLA-A, -C, -B, -DRB1, -DQB1, and -DPB1 typing is performed for parents and five children. Haplotype segregation demonstrates three key concepts. First, parental haplotypes "c" and "d" differ only at HLA-DPB1. Six-locus typing is needed to discriminate two different haplotypes. Second, Child #1 and Child #2 are identical-by-descent and are "HLA genotypically-matched siblings". Finally, Child #3 and Child #5 are haploidentical to each other due to two different A1-C7-B8-DR3-DQ2 haplotypes (haplotypes "c" and "d"). Their haploidentity can be confirmed by typing HLA-DPB1.

Haplotypes are central to the selection of a donor when a second haploidentical transplant is required to treat relapse. This concept is discussed in detail in Section 11.4.8 "HLA Loss". Haplotypes *per se* have functional meaning in transplantation beyond patient/donor mismatching. Jean Dausset described conserved haplotypes in the French population in 1978 [82], and evaluation of "ancestral haplotypes" opened a new field of immunogenetics in which linked factors could play a role in autoimmunity [83–86]. Conserved extended haplotypes are long-range, often measuring megabases (Mb) in distance. Positive linkage disequilibrium (LD) is seen when the frequency of the specific combination of allotypes in the population exceeds that which is expected based on frequencies of the individual determinants

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[87, 88]. Early studies of recombination used microsatellites to characterize the organization of the class II region [89] and later extended characterization to the class III region [90].

Sequencing of HLA haplotypes provides the highest resolution definition of the content and organization of variation. In 1999, the MHC Sequencing Consortium published the first complete sequence and gene map of the MHC [91]. The map spanned 3.6 Mb, identified 224 genetic loci, and represented a compilation of different haplotypes that were assembled from a core group of laboratories. This first-generation map provided an unprecedented view into the patterns of simple and more complex forms of variation. The complete sequencing of common HLA haplotypes soon ensued [92, 93] and provided over 4 Mb of contiguous sequence for common haplotypes including HLA-A1-C7-B8-DR3, A3-C7-B7-DR15, and A26-C5-B18-DR3-DQ2. In 2008, the MHC Haplotype Project used an antigen-homozygous haplotype as an annotated reference sequence for comparative evaluation of haplotypes observed commonly in European populations [94]. Next-generation sequencing methods have extended analysis of HLA haplotypes [95], and recent efforts have shed new light on the C4 region in particular [96].

The contributions made by the HLA community in establishing reference haplotypes with complete sequence information provided the foundation for subsequent evaluation of MHC-region variation for the purposes of mapping disease genes [97, 98]. Gene mapping platforms are descriptive of the simplest form of human genetic variation, the single nucleotide polymorphism (SNP). Such genotyping approaches are designed to query the extent of variation within the human genome using well-characterized "tagSNPs". TagSNPs are SNPs that are in complete LD with one or more SNPs that reside within a given haplotype block. Genotyping of tagSNPs provides information on the untyped SNPs within that block [99]. These approaches have been instrumental for locating genes that cause disease [98]. MHC-specific tagSNPs were used to identify regions of the MHC that harbor functional transplantation determinants [100, 101], and fine-mapping of SNP haplotype blocks has yielded new information on the mechanisms of HLA alloreactivity in HCT [102, 103].

11.3.1.1 Clinical Significance of Haplotypes

Haplotypes have been instrumental in identifying new features of HLA allotypes that play a role in transplant outcomes. The rs429916A/C SNP was validated as a marker for mortality in HLA-mismatched unrelated donor HCT [101]. Fine-mapping across the class II region localized the causative protein to be HLA-DR and specifically a motif encoded by residues 26-28-30 of HLA-DRβ1 most commonly found on class II haplotypes in African and Hispanic American populations [103]. This experience highlights the concept that the causal gene may reside at great distances from the original SNP marker due to long-range LD.

Haplotypes are not only informative for HLA allotypes but also for variants that regulate the expression of HLA [104, 105]. Haplotype-specific patterns can be described for transcriptional regulatory elements, CG sites that alter methylation, and expression quantitative trait loci [104, 106].

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Haplotypes can provide information on GVHD risk. The Japanese population has conserved haplotypes some measuring upward of 7 Mb in length [107]. This striking degree of genetic fixity enabled analysis of three major extended haplotypes in a cohort of unrelated donor transplants from Japan: HLA-A*24:02-C*12:02-B*52:01-DRB1*15:02-DQB1*06:01-DPB1*09:01 ("P1"); HLA-A*33:03-C*14:03-B*44:03-DRB1*13:02-DQB1*06:04-DPB1*04:01 ("P2"); HLA-A*24:02-C*07:02-B*07:02-DRB1*01:01-DQB1*05:01-DPB1*04:02 ("P3"). Possession of P3 was associated with significantly higher risk of acute GVHD, whereas P1 was protective. An updated survey of haplotypes demonstrates the biological significance of common haplotypes in Japanese patients [108].

Haplotypes may also provide information on untested variation, and the potential that patients and donors who appear to be phenotypically identical may be genotypically mismatched. In HLA-10/10-matched unrelated donor HCT, patients and donors with different HLA-A-B-DR haplotypes had significantly higher risk of severe acute GVHD compared to pairs with the same three-locus haplotype [109]. "Haplotype matching" might predict matching not only for the known HLA loci, but for untyped variation linked to those markers. Further evidence that haplotypes carry clinical significance has been shown among HLA-mismatched unrelated donor transplants where haplotype disparity was associated with mortality [110]. Untyped haplotype-linked variation might be contributed by any gene with immune-related function, including class III and class I determinants [111, 112].

11.3.2 Role of Classical HLA Loci in Haploidentical Related Donor Transplantation

Much of our understanding of the importance of donor matching has come from extensive analyses in HLA-mismatched unrelated donor HCT, where the classical definition of graft-versus-host and host-versus-graft disparity has been validated for GVHD and graft rejection, respectively. The unrelated donor transplant experience has also shown that risks are imparted not only by disparity but also from the classic disease-association model in which possession of certain molecules or epitopes may themselves be immunogenic and increase risk. A newer concept in allogeneic transplantation is that not all mismatches produce higher risk; there is reproducible evidence in the haploidentical related donor transplant model demonstrating convincing protective effects of disparity (Table 11.3). The reader is invited to a discussion of HLA in unrelated donor HCT in Chapter 12.

Complications after haploidentical related donor HCT do not appear to correlate with the number of HLA-mismatched loci on the non-shared haplotype [38, 117–121]. The effects of HLA disparity on clinical outcome may depend on the transplant protocol and use of immunosuppressive agents [120]. As the number of transplantations performed with PTCy increases, more data have become available and show the impact of HLA mismatching on the success of haploidentical related donor HCT [113, 114, 119, 120]. Furthermore, the classical teaching that HLA mismatching increases risks may be different in haploidentical related donor HCT with PTCy, as shown for HLA-DRB1 discussed below [38, 113, 114].

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Table 11.3 Genetic factors associated with clinical outcome in haploidentical related donor transplantation.

Locus	Effect	References
HLA-B	HLA-B leader matching lowers relapse and improves disease-free and overall survival. HLA-B leader mismatching with DRB1-matching is associated with a high risk of relapse.	[113]
HLA-C	HLA-C allele mismatching increases the risk of chronic GVHD.	[113]
HLA-DRB1	HLA-DRB1 mismatching lowers the risk of relapse. Its effect is synergistic with DQB1-matching and HLA-B leader-matching.	[38, 113, 114]
	Patient HLA-DRβ Gly86Val genotype is a germline risk feature.	[115]
HLA-DQB1	HLA-DQB1 allele matching is associated with lower transplant-related mortality. HLA-DQαβ heterodimers composed of DQα 01 with DQβ 05/06 are associated with a higher risk of relapse especially if it is the mismatched molecule in the patient.	[115]
HLA-DM	HLA-DMA*01:01-DMB*01:01 molecules are associated with lower relapse than other DMαβ heterodimers.	[115]
HLA-DR-DQ-DM Haplotype	Patient and donor haplotypes provide information on risks.	[115]
HLA-DPB1	T-cell epitope nonpermissive mismatching is associated with lower relapse.	[113]
MICA	Donor Met129Val matching and exon 5 STR mismatching is associated with lower relapse.	[116]
MICB	Patient MICB residue 52 is associated with lower relapse.	[116]

Finally, the MHC is residence to HLA molecules that serve as ligands for NK receptors, and new data demonstrate that the clinical significance of HLA ligands in transplantation requires knowledge of the donor receptor. These concepts are now more fully explored below.

11.3.3 The Non-shared Haplotype

The non-shared haplotype contributes matched or mismatched HLA allotypes. The HLA-DR-DQ segment provides the best example of how loci that have traditionally been considered to increase risk in the mismatched state in unrelated donor HCT may show dis-synchronous effects in haploidentical related donor HCT. HLA-DRB1 mismatching conferred protective effects on relapse and overall survival in several single-center studies [38, 114] and was recently confirmed in

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a retrospective registry-based observational study of patients who received PTCy for GVHD prophylaxis [113]. Interestingly, HLA-DQB1 matching together with HLA-DRB1-mismatching further enhanced the protective effects of HLA-DRB1 disparity, improving disease-free survival by 20% compared to HLA-DRB1- and HLA-DQB1-mismatching. The second locus to display protective effects with mismatching was HLA-DPB1. HLA-DP T-cell epitope (TCE) nonpermissive mismatches were associated with improved overall survival. These data show that not all HLA mismatches impart higher risk relative to matching. Second, in the case of HLA-DR and -DQ, there may be interactive effects and individual locus effects may be in opposing directions.

The HLA-B leader peptide is a newer model of alloreactivity, first described in unrelated donor HCT and cord blood transplantation [122–124]. In the same US registry cohort described above, an HLA-B allele mismatch as defined classically in the GVH vector was not associated with any clinical endpoint. When the patient and donor's mismatched HLA-B alleles were defined by their leader peptides, HLA-B mismatches that were leader-matched (alleles of the same leader clade) were associated with significantly lower relapse and improved survival compared to leader-mismatched allele mismatches (alleles from different leader clades), mirroring the findings in the unrelated donor experience. Since mismatched HLA-B and HLA-DR are co-inherited on the non-shared haplotype, an analysis of HLA-B leader (mis)matching with HLA-DRB1 (mis)matching was undertaken and revealed additive effects of the leader and HLA-DRB1. The optimal haploidentical donor from the perspective of HLA-B and -DR is one who is leader-matched but HLA-DRB1-allele mismatched. Finally, despite its relatively lower overall level of expression compared to other class I, HLA-C mismatches in the US haploidentical related donor cohort increased the risk of chronic GVHD. Taken together, this registry-based study provides evidence that, despite low overall acute GVHD rates in PTCy-based haploidentical related donor HCT, HLA (mis)matching influences clinical outcomes in consistent and reproducible ways.

11.3.4 NIMA

One of the first descriptions of the functionality of HLA haplotypes pertains to non-inherited maternal antigens (NIMAs) [125]. The most pronounced beneficial effect of NIMA has been in renal transplantation where the underlying mechanism is the establishment of microchimerisms of NIMA-expressing donor cells which maintain a tolerant state in the patient [126]. In haploidentical related donor HCT, transplantation from mothers as donors is associated with higher survival and lower probability of non-relapse mortality, with no difference in the risk of severe acute GVHD or relapse, compared to transplantation from fathers as donors [127].

NIMAs are tolerizing in haploidentical related donor HCT for regimens that do not include PTCy [128–130]. This means that transplantation from mother to child is associated with a lower risk of both acute and chronic GVHD and mortality, indicating donor-specific suppression of T-cell responses against NIMAs. In contrast, transplantation from father to child is associated with a higher risk of

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GVHD, suggesting an immunizing effect of paternal antigens. The potential effect of NIMA in the setting of haploidentical related donor HCT with PTCy remains an area for investigation.

11.3.5 Unique Functional Features of Class II Molecules

The class II region presents several unique structural features that have been known to play important roles in autoimmunity [131–134]. Each class II molecule is an αβ heterodimer formed by an α chain encoded by A genes (DRA, DQA1, DMA, DOA, and DPA1) and a β chain encoded by B genes (DRB1,DRB3, DRB4, DRB5, DQB1, DMB, DOB, and DPB1). A recent analysis of class II in haploidentical transplantation evaluated HLA-DR, -DQ, -DM, and -DO molecules and haplotypes [115]. HLA-DRA shows sequence diversity albeit at a far lower level than HLA-DRB1 [135]. Two SNPs are of interest and include the exon 3 rs8084 dimorphic SNP known to generate transcripts of different lengths, and the exon 4 rs7192 Val217Leu missense change. Neither SNP correlated with transplant outcomes in the patient, the donor, or patient/donor mismatching.

In contrast to HLA-DRα, HLA-DRβ1 was associated with striking differences in relapse and survival. The Val86Gly dimorphism is best characterized for its role in autoimmunity, in which it is hypothesized that the substitution may modulate the size of peptide residue side chains that can be accommodated in the pocket [136–138]. Although there are over 3,600 unique HLA-DRB1 alleles, each DRβ1 encodes either an 86Val or 86Gly. Among a cohort of 1,629 patients transplanted from haploidentical related donors, the majority of whom received PTCy for GVHD prophylaxis [115], the risk of posttransplant relapse among HLA-DRB1-matched pairs was lowest for patients who were heterozygous for DRβ-86ValGly relative to GlyGly patients. Among HLA-DRB1-mismatched patients, the risk of relapse was lowest for DRβ-86GlyGly patients transplanted from DRβ-86GlyVal donors. The findings extend the previous registry observations that relapse risk is lower with DRB1 allele mismatching, but the protective effect is most clearly observed in HLA-DRB1-allele mismatched GlyGly patients/GlyVal donors.

The functional significance of the HLA-DQ αβ heterodimer was initially described in HLA-DQB1-allele mismatched unrelated donor HCT [139]. Based on surface expression of DQα-DQβ heterodimers, crystallographic structure, and peptide/HLA-DQ functional data [140, 141], two groups of HLA-DQ molecules have been defined. Group 1 ("G1") comprises heterodimers of any DQα 02, 03, 04, 05, or 06 with any DQβ 02, 03, or 04. Group 2 ("G2") molecules are formed by DQα 01 with any DQβ 05 or 06. In HLA-DQB1-allele-mismatched unrelated donor HCT, the presence of a G2 molecule increased the risk of relapse and lowered disease-free survival; risks were further increased if the G2 molecule in the patient was mismatched with the unrelated donor's HLA-DQ. Similar G2-associated risks were observed among haploidentical related donor transplants. G1G2 patients transplanted from G1G1 haploidentical donors had lower disease-free survival compared to HLA-DQ-matched transplants, recapitulating the observations in the unrelated donor transplant setting.

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The MHC class II region encodes the classical class II antigen-presenting molecules HLA-DR, -DQ, and -DP, but also accessory molecules HLA-DM and -DO which play an important role in regulating the loading of peptides from self- and foreign-antigens into class II molecules [142]. Whereas HLA-DM facilitates the exchange of peptides required for loading of peptides into class II, HLA-DO modulates HLA-DM function. HLA-DMA, -DMB, -DOA, and -DOB are polymorphic, with at least 59, 82, 93, and 63 unique alleles, respectively [135]. To better understand their clinical significance in haploidentical related donor HCT, HLA-DM and -DO were characterized for α- and β-chain variation, and subsequently αβ heterodimers [115]. Neither HLA-DOα, -DOβ, nor DOαβ heterodimers provided information on transplant outcomes. However, HLA-DMα and DMβ variation informed relapse and survival, where missense changes at HLA-DMα-184 and -155 and DMβ-10 correlated with relapse, disease-free survival, and mortality. As an HLA-DMαβ heterodimer, molecules comprised of DMA*01:01-DMB*01:01 ("DM11") were associated with superior disease-free survival.

With the identification of functional features for HLA-DR, -DQ, and -DM, it was feasible to define three-locus HLA-DR-DQ-DM αβ haplotypes in the clinical cohort. Not only did the patient's and the donor's non-shared haplotypes influence disease-free survival, but risk for a given haplotype depended on whether it was present in the patient or the donor.

Taken together, these findings indicate that HLA determinants may function as individual allo-determinants and as haplotypes of linked molecules whose overall effects might reflect complex interactions and synergisms across MHC haplotypes.

11.3.6 The Innate Immune System

The innate immune system plays a primary role in the control of viral infection and tumor killing [143]. Most of the data have shed light on the genes of the killer immunoglobulin-like receptors encoded on chr 19q13.4. Models of KIR alloreactivity in allogeneic HCT have been demonstrated to impact relapse risk after transplantation. Although more information has been available for the role of KIR in matched or mismatched unrelated donor transplantation [144–150], the original landmark observations were made in patients undergoing haploidentical related donor HCT and tested the KIR ligand mismatch model as a major mechanism of NK-mediated graft-versus-leukemia [151]. These discoveries inspired both basic and clinical studies of the innate immune system in transplant immunobiology. The reader is invited to Chapter 5 for a comprehensive review of the KIR genetic system.

11.3.6.1 KIR in Hematopoietic Cell Transplantation

There are several models for KIR in transplantation. The patient's germline HLA contributes the ligands for donor KIR, and therefore, the mechanisms depend on whether the patient and donor are HLA-matched or HLA-mismatched. In HLA-matched patients and donors who are homozygous for HLA-C1C1 or C2C2 or HLA-Bw6,Bw6, the ligand that is missing in the patient may release inhibition of donor NK cells that express the inhibitory cognate receptor for that missing ligand,

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and lead to cytotoxicity of the patient's leukemia, leading to lowered relapse and improved survival [147, 152, 153].

Haploidentical related patients and donors present mismatched ligands from the non-shared haplotype. In the KIR mismatch model, donor NK alloreactivity against patient leukemia cells is provoked when there is a ligand mismatch in the graft-versus-host vector of incompatibility. Alloreactive clones directed against patient leukemia cells lead to lower relapse after T-cell depleted (ex vivo) haploidentical HCT [154, 155]. Importantly, patients did not have higher GVHD risk, and the benefits of this transplant approach were therefore three-fold: improved engraftment, low GVHD, and robust graft-versus-leukemia effects. The beneficial effect of KIR ligand mismatching is also present in patients undergoing PTCy-based haploidentical related donor HCT for relapsed disease, in whom KIR ligand mismatching is associated with lower relapse and improved progression-free survival; this beneficial effect was not observed in patients transplanted in a remission of their leukemia [156]. The benefits of lower relapse and rates of infectious complications are seen when donors express the activating KIR2DS1 or KIR3DS1 receptors in KIR ligand mismatched haploidentical related donor HCT [157].

Lower relapse has also been observed when the patient and haploidentical related donor both have C1, C2, Bw4, and all donor inhibitory KIR [158]. PTCy in unmanipulated haploidentical HCT effectively abolishes alloreactive T cells, thus dramatically lowering acute GVHD. However, PTCy also eliminates mature NK cells that express inhibitory KIR receptors, leading to loss of protection from leukemia relapse [159, 160].

A third model for KIR allorecognition in HCT is the contribution of the multigene KIR haplotypes. KIR genes are co-inherited on haplotypes named "A" and "B" that differ for the number of activating/inhibitory KIRs. In children with ALL, clinical outcomes after T-cell depleted haploidentical related donor HCT were associated with the number of B motifs on the donor haplotypes. Transplantation from donors with B haplotypes (indicative of activating KIR haplotypes) was associated with lower relapse and better event-free survival compared to donors with A haplotypes [161]. Notably, higher numbers of centromeric and telomeric KIR B motifs were associated with a significant reduction in relapse and could be quantified using a score for B haplotype content [161].

The favorable effects of donor KIR B haplotypes are best observed in T-cell depleted haploidentical related donor transplantation, which support the selection of haploidentical donors with alloreactive NK cells [157, 161, 162]. In T-replete haploidentical related donor transplantation, the data are more heterogeneous. Whereas the findings of some studies suggest a potential benefit to selecting haploidentical donors based on predicted NK alloreactivity defined by KIR mismatching or haplotypes [118, 156, 163], other cohorts are less conclusive [164–167].

11.3.6.2 Nonclassical Class I HLA Ligands for Donor NKG2 Receptors

The majority of data regarding the biology of nonclassical class I loci have been gleaned from the unrelated donor transplant experience, and the reader is invited to the presentation in Chapter 7. New data have emerged on the role of MICA and

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MICB and their NKG2D receptor in haploidentical related donor HCT [116]. MICA and MICB are stress ligands and display sequence variation with over 530 unique MICA and 245 unique MICB alleles [135]. Their receptor, NKG2D, is expressed by NK cells, cytotoxic αβ $CD8^+$ T cells, and γδ T cells, and, in this way, may broadly participate in T- and NK-cell alloresponses in transplantation [143].

Early interest in MICA in transplantation stemmed from the observation that the Met129Val missense change is strongly associated with different binding affinities of the ligand to NKG2D [168]. In retrospective analysis of HLA-10/10-matched unrelated donor transplants, MICA matching was associated with lower risks of GVHD without any effect on survival [169]. The beneficial effects of MICA matching were confirmed in additional studies of matched and mismatched unrelated donor transplants in some [170] but not other studies [171]. A SNP mapping approach yielded evidence for donor MICA-129Met and lower incidence of severe acute GVHD [172].

Less well understood has been the role of MICA exon 5 sequence diversity in transplantation. Exon 5 encodes a short tandem repeat (STR) that generates 4–10 alanine residues in the transmembrane portion of MICA molecules. Exon 5 also encodes rs67841474G, a single base insertion that is present in selected alleles termed "A5.1". This SNP introduces a premature stop codon that results in a truncated short protein that lacks the transmembrane and cytoplasmic tail. These structural differences are associated with changes in surface and soluble expression and altered cytotoxicity [173]. The clinical significance of these exon 5 polymorphisms is not known.

Less information has been available for MICB. MICB has four higher frequency missense changes: residues Asp52Asn, Lys57Glu, Ile98Met, and Thr189Ile. In a study of HLA-10/10-matched unrelated donor transplants, patient/donor mismatching for MICB Ile98Met was found to increase the risks of both severe acute GVHD and relapse [174].

To better understand the potential functional significance of MICA, MICB, and NKG2D in haploidentical related donor HCT, each gene was studied in a cohort of patients who received PTCy-based regimens [116]. The MICA Met129Val dimorphism did not correlate with any clinical endpoint in the patient, the donor, or patient/donor mismatching. However, the MICA exon 5 STR showed strong associations in which a GVH mismatch was associated with improved disease-free and overall survival. The beneficial effect of STR-mismatching was further enhanced when the patient and donor were residue 129-matched. These results are an example of a protective effect of mismatching and synergism between different features within the MICA molecule.

Of the four missense changes in MICB, residue Asn52Asp (rs3131639 A/G) informed mortality, where the AA genotype (52Asn) increased mortality compared to the GG genotype (52Asp). The model proposed that the recipient's cells present MICA and MICB ligands which are recognized by donor NK and/or T cells that express the NKG2D receptor. The NKG2D sequence is relatively conserved with a single missense residue Ala72Thr in the cytoplasmic domain. Although this missense substitution is not predicted to directly alter ligand/receptor interactions, it is a putative expression quantitative trait locus. Patients transplanted from

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haploidentical donors with NKG2D 72ThrThr had superior survival compared to patients with 72AlaAla or AlaThr donors.

MICA, MICB, and NKG2D inform clinical outcomes. MICB represents a patient germline feature that cannot be modified, but MICA and NKG2D are donor-specified risk factors which might be optimized if a patient has a choice of haploidentical relatives. These concepts motivated the joint consideration of patient MICB ligand with donor MICA STR-mismatching and the NKG2D-72 receptor polymorphism, and provided the most robust correlation with relapse. Among patients with favorable MICB-52Asp, relapse risk was further lowered with transplantation from donors who were both MICA STR exon 5-mismatched and NKG2D-72Thr. Collectively, these findings support a model in which consideration of the ligand with its receptor provides optimal information that can be used to select haploidentical donors to lower the risk of posttransplant relapse.

11.3.7 HLA Sensitization

HLA sensitization is the chief cause of graft rejection, and patients planning a haploidentical related donor HCT should regularly undergo anti-HLA antibody screening, particularly patients who require significant transfusional support [175]. EBMT and the British Society for Histocompatibility and Immunogenetics (BSHI) have provided guidelines for the detection of donor-specific anti-HLA antibodies [176, 177]. A presentation of rejection and antibodies is presented in Chapter 9.

Anti-HLA antibodies directed against the mismatched donor HLA antigens delay engraftment and result in poor graft function and graft rejection [178–184]. Graft rejection occurs from antibody-dependent T-cell mediated cytotoxicity or complement-mediated cytotoxicity, and the risk factors are median florescence intensity (MFI) values greater than 3,000 and C1q positivity [180, 181, 183]. When patients have multiple haploidentical relatives to choose from, selection of a donor with "safe" antigens (patient lacks anti-donor HLA antibodies to the mismatched antigens) is preferred [177, 185]. In the absence of donor choices, patients have successfully engrafted following pretransplant desensitization [26, 176, 186–190].

A special circumstance is presented when the mother can serve as the haploidentical transplant donor for a child. Data in T-cell depleted haploidentical HCT suggests that knowledge of NIMAs [191] may be helpful for donor selection. A child will tolerate mismatched maternal antigens more so than mismatched paternal antigens due to microchimerism while in utero; the tolerizing effect of mismatched maternal antigens is also associated with lower relapse, lower non-relapse mortality, and improved survival after T-cell depleted haploidentical HCT [192, 193]. When several DSA-free donors are available, selecting a donor based on NIMAs may be beneficial.

11.3.8 HLA Loss

Graft-versus-host disease is a clinical manifestation of donor alloreactivity against patient HLA antigens expressed on host tissues that are not present in the donor. Donor recognition of patient HLA expressed on residual leukemia cells is required

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for the graft-versus-leukemia (GVL) effect. Both clinical scenarios depend on recognition by the donor of expressed patient HLA. When the mismatched HLA antigens of the patient are no longer expressed on leukemia cells, there are no targets against which the donor can mount a GVL effect. The loss of HLA expression on patient leukemia was recognized as a mechanism of posttransplant relapse [194] and has furthered understanding of the immunology of relapse in allogeneic transplantation. There is a growing body of literature on HLA loss, and the reader is invited to outstanding reviews in this area [26, 195–197].

Overall clinical experience demonstrates that the risk of relapse after allogeneic HCT ranges from 10 to 30% and depends on the stage of the underlying disease at the time of conditioning, the risk features of the disease, transplant regimen, and donor source. The loss of HLA expression on the surface of residual patient leukemia cells is estimated to occur in up to 30% of patients who relapse after HCT [194, 198–201]. HLA loss has been observed after haploidentical related donor HCT with a variety of transplant regimens including the use of methotrexate/ATG, PTCy, and αβ T-cell/CD19-depleted allografts [195, 198, 202]. Demographic characteristics of patients with relapse and HLA loss include those with active disease at the start of conditioning and chronic GVHD [195, 199].

11.3.8.1 Mechanisms

There are at least three mechanisms that can lead to the loss of HLA. Acquired somatic uniparental disomy (aUPD) is one mechanism in which there is genomic loss of HLA. The scale of the loss may be limited to one locus, or large-scale involving the entire mismatched HLA complex on the non-shared haplotype. Loss can involve class I and/or class II. The loss of the genomic region occurs during mitosis, and the lost region is replaced by a copy from the intact haplotype. This results in no change in the genomic copy number ("copy neutral") and acquired homozygosity ("loss of heterozygosity"). This is the basis of the term "copy neutral loss of heterozygosity" (cnLOH). cnLOH HLA loss may be observed in up to 30% of patients who relapse after transplantation, particularly patients with active leukemia at the time of conditioning [198, 201, 203]. Data also show that the number of T cells in the infused allograft is correlated with risk of cnLOH after T-replete haploidentical related donor HCT [199]. Possible mechanisms of cnLOH in HLA-mismatched HCT include immunological pressure applied on patient leukemia cells by donor alloreactive T cells which promote immune escape [204]. Although originally described in the setting of haploidentical related donor HCT with one complete haplotype mismatch, HLA loss and relapse have also been observed in HLA-matched and HLA-mismatched unrelated donor HCT and raise interesting possible mechanisms including the recognition of minor histocompatibility antigen differences and whether some HLA mismatches promote HLA loss to a greater degree than other mismatches [205–208].

A second mechanism of the loss of HLA class II antigens is epigenetic downregulation of the CTIIA class II transactivator [209, 210]. This mechanism may account for approximately 40% of patients who relapse after HCT. Since downregulation may involve matched or mismatched class II antigens, it is a mechanism in both

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HLA-matched and -mismatched HCT. The chief pathway for downregulation of CTIIA is through hyper-methylation of the promoter [210] and has been linked to the use of peripheral blood stem cell sources and the dose of T cells in the graft [209]. A role for minor histocompatibility antigens as a potential driver of immune pressure is hypothesized because HLA mismatching is not a requisite [211, 212]. As described in the next section, recombinant human interferon-γ may reverse the downregulation and restore donor T-cell allorecognition [209, 210].

A third mechanism used by leukemia cells is upregulation of T-cell inhibitory ligands PD-L1, B7-H3, CD80, and PVRL2 which inhibit donor T-cell responses particularly after haploidentical related donor HCT [209, 213–217]. The expression of inhibitory receptors can be exaggerated in the setting of allogeneic HCT and provoked by the donor's immune system [218]. Downregulation of class II with this mechanism is observed after haploidentical relate donor HCT as well as HLA-matched transplantation.

11.3.8.2 Laboratory Detection of HLA Loss

Chimerism testing in the posttransplant period is routinely performed to quantify the relative amounts of donor and recipient-derived cells (please refer to Chapter 13 for detailed presentation on chimerism) and plays an important role in the evaluation of relapse. HLA loss can be ascertained at the time of relapse with several techniques that provide complementary information [219]. Often several approaches are combined to secure the diagnosis of HLA loss, as each method has its advantages and disadvantages. At the core of the evaluation for HLA loss is a repeat of HLA typing, and there are a variety of molecular platforms in routine use in clinical laboratories [202, 203, 220, 221]. A test of a buccal swab of the patient can serve as a standard for confirming the patient's germline HLA [222, 223]. Chimerism testing can readily be used to measure HLA loss in patients who relapse [224–229]. Inclusion of markers for the HLA region, together with HLA typing, has high sensitivity and accuracy. Different methods for chimerism testing have been successfully used including short tandem repeat (STR) analysis, and depending on the adequacy of coverage of the HLA region, STR-based chimerism assays can be combined with HLA testing [194, 230, 231]. Array-based platforms capture large regions for the detection of loss of heterozygosity within the HLA region [194, 203, 206–208].

11.3.8.3 Treatment

The treatment of relapse after haploidentical related donor HCT depends on the mechanism of HLA loss. If cnLOH is documented, a second transplant with a different donor may be considered. The selection of a haploidentical donor for a second transplant is based on the patient's mismatched haplotype that is lost. Figure 11.1 serves to illustrate a hypothetical situation of a patient (Parent #1) who relapses after a transplant from a haploidentical donor (Child #1). The patient loses haplotype "b", the haplotype that was mismatched with haplotype "c" of the first donor. The appropriate donors for consideration for the second transplant include Child #3 or Child #5. Child #3 and Child #5 share the lost "b" haplotype with the patient (and therefore would not increase GVHD for non-hematopoietic-cell

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tissues that express allotypes from haplotype "b"), but they are mismatched against the patient's retained "a" haplotype whose HLA allotypes are expressed on the relapsed leukemia cells. The mismatch between haplotypes "a" in the patient and "d" in Child #3 is expected to mount a graft-versus-leukemia attack. The same principle holds for the mismatch between haplotypes "a" in the patient and "c" in Child #5. Child #4 matches the patient for haplotype "a", the patient's retained (expressed) haplotype. Since the leukemia cells have lost expression of allotypes from haplotype "b", Child #4 would not mount a graft-versus-leukemia attack; moreover, non-hematopoietic cell tissues that express allotypes from haplotype "b" might be seen as foreign, leading to unwanted GVHD.

For relapses in the setting of *non*-cnLOH, interferon-γ has been used to increase class II expression in the case of epigenetic downregulation of class II [196], and checkpoint blockade when there is up-regulation of inhibitory molecules albeit with potential for increasing GVHD [232, 233].

11.4 Selection of Related Donors for Transplantation: Non-HLA Factors

As described in Section 11.2.1, patient characteristics (for example, disease risk; patient functional score) strongly influence the timing of transplantation as well as how the transplant will be performed (for example, the intensity of the conditioning regimen, manipulation of the graft, GVHD prophylaxis) which are related to the type of donors available. The non-HLA factors that are considered in the selection of haploidentical related donors have been extensively studied in single center and registry analyses [26, 117, 118, 120, 185, 234, 235]. The importance of individual donor factors in the success of the transplant is well-described; however, data are still needed to understand the composite impact of the constellation of factors that can define the "optimal" haploidentical donor. Until then, maximizing the favorable factors while avoiding or minimizing unfavorable features (for example, donor-specific HLA antibodies), to the extent that is possible, can assist in the prioritization among otherwise equivalent haploidentical relatives (Table 11.4). First degree haploidentical relatives are usually considered; however, haploidentical second degree relatives have provided life-saving stem cells [236]. For pediatric patients, suitable donors may include parents, but may depend on NIMA and the transplant procedure to be used [117]. For older patients, suitable donors may include children. NIMA, sex, and age are linked factors. For example, mothers who serve as donors provide a tolerizing effect, but are also older. Future research is needed on how new MHC resident variation such as MICA, MICB, HLA-DM, factor into donor selection in relation to non-HLA factors.

The role of family typing is to both identify suitable transplant donors and to establish haplotypes. Screening of family members with the aid of health questionnaires may assist in planning the family typing, and to identify family members with health concerns that may make them ineligible to serve as donors. Resolution of HLA-A, -C, -B, -DRB1/3/4/5, -DQA1, -DQB1, -DPA1, -DPB1 alleles

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Table 11.4 Factors influencing the selection of haploidentical donors for transplantation.

Factor		Model	Favorable
Classical HLA	HLA-B leader	Patient/donor match	Match
	HLA-C	Patient/donor match	Match
	HLA-DRB1 allele	Patient/donor match	Mismatch
	HLA-DRβ Gly86Val	DRB1-matched patient and donor	GlyVal
		DRB1-mismatched patient and donor	GlyGly patient with GlyVal donor
	HLA-DQB1 allele	Patient/donor match	Match
	HLA-DQ αβ heterodimer	Patient genotype	Lack of G2 heterodimer
		Patient/donor match	Lack of mismatching against patient G2
	HLA-DPB1	Patient/donor match	T-cell epitope nonpermissive mismatch
Nonclassical HLA	MICA	Patient/donor match	Met129Val match and STR mismatch
	MICB	Patient genotype	Residue Asp52Asn
	NKG2D	Donor genotype	Residue Ala72Thr
	HLA-DM	Patient; donor	Presence of DMA*01:01-DMB*01:01 heterodimer.
Donor-specific antibodies		Patient anti-donor HLA-specific antibodies	None; MFI < 1,000
Donor Age	—	—	Inconclusive. If there is a choice: younger
Donor Relationship	—	—	Inconclusive. If there is a choice between parents: mother (NIMA). If the choice is between multiple generations: younger.
Donor Gender	—	—	Inconclusive. If there is a choice: male donor for male patient.
CMV	—	Patient/donor match	CMV-negative donor for a CMV-negative patient
ABO	—	Patient/donor match	Match or minor mismatch

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provide information on haplotypes and haploidentical family members who shared one complete haplotype (Figure 11.1; [177, 186]). Until recently, non-HLA factors figured prominently into donor selection (with the exception of screening for donor specific anti-HLA antibodies). With the recent data pointing to a protective effect of HLA-DRB1-mismatching/-DQB1-matching and HLA-B leader matching of the non-shared haplotype, their consideration might be feasible if there are two or more otherwise equivalent donors.

The beneficial effects of younger donors is seen in some allogeneic transplant cohorts but not others, and the favorable effect may depend on the age of the patient [117, 118, 234, 237–245]. Consideration of gender of the donor may depend on patient gender, the preference for NIMA and on the specific transplant regimen used [38, 117, 185, 245, 246]. In general an ABO-matched donor is preferred over a donor with a major ABO-mismatch to optimize engraftment; transplant-associated risks associated with minor ABO-mismatched donors may depend on the graft source (marrow versus peripheral blood stem cells) [247, 248]. The role of donor CMV serostatus in unrelated donor transplantation is well-defined [249]. The data in haploidentical related donor HCT are more heterogeneous [118, 234, 250, 251].

11.5 Concluding Remarks

The field of haploidentical related donor HCT has witnessed tremendous advances in the basic understanding of the transplantation barrier. Indications for the timing of transplantation depending on disease diagnosis, disease stage, and patient fitness provide the opportunity to plan transplantation at a time when the benefits of curative therapy greatly outweigh the risks from the transplant procedure itself. The age of patients who benefit from HCT can be extended with the use of less intense conditioning regimens. Acute GVHD risk can be significantly lowered with T-cell depletion or T-replete multi-agent immunosuppressive prophylaxis regimens. The ideal haploidentical donor is an on-going area of clinical research. Data thus far show that many of the genetic associations previously observed in unrelated donor HCT with traditional calcineurin inhibitors for GVHD prevention, are also seen in haploidentical related donor HCT using PTCy. Since the first haploidentical transplantations were performed in the 1990s, the availability and safety of haploidentical HCT have improved tremendously. The primary cause of transplant failure today remains relapse, and on-going research efforts aimed at understanding the mechanisms of disease relapse and appropriate directed therapies, will continue to shed light on this important clinical endpoint. Continued efforts to understand how patient, donor and transplant factors interact with each other will help to clarify the optimal donor and transplant regimen that meet the needs of each unique patient and the preemptive measures to prevent relapse.

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12

Stemcell Transplantation from Unrelated Donors

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12.1 Introduction

Stem cell transplantation (SCT) is an advanced therapeutic modality that has transformed the treatment of various life-threatening diseases, including certain cancers, genetic disorders, and autoimmune conditions. At its core, SCT aims to restore a patient's immune system and hematopoietic function by infusing stem cells that can regenerate the blood and immune cells. SCT can be performed using either autologous (patient's own) or allogeneic (donor-derived) stem cells. Among the allogeneic options, unrelated stem cell transplantation plays a pivotal role, particularly when a favorable related donor is not available. This is increasingly common in Western countries, where large families with one genetic background are becoming rare and most patients are older, making siblings as donors unavailable [1].

When a related donor is not available, an unrelated donor is sought, often from large international registries. Matching the donor's human leukocyte antigen (HLA) types with the recipient's is critical to minimizing the risks of immune rejection and graft-versus-host disease (GVHD) [2]. Due to the growing number of global registries, the chances of finding a suitable unrelated donor have increased, though mismatches still pose a significant challenge in some cases, particularly among ethnic minorities [3].

12.2 The Search for Unrelated Donors

Unrelated stem cell transplantation relies on the availability of a suitable donor, which is typically identified through international donor registries. These registries collect genetic information from potential donors to match them with patients who require a transplant.

The global search for an unrelated stem cell donor is organized and coordinated by a combination of national and international organizations. Here's how the global search works, along with the key players involved.

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12.2.1.1 National Registries

Each country typically has its own national donor registry, which maintains a list of potential stem cell donors. Some of the major national registries include

Europe and the United States

- Be The Match (National Marrow Donor Program) in the United States
- Anthony Nolan in the UK
- ZKRD (Germany, focus on IT-technical, security of the search and data safety)
- DKMS (Germany, Poland, and other countries; focus on medical purpose)
- Agence de la Biomédecine (France)
- France Greffe de Moelle
- Belgian Bone Marrow Registry (Belgium)
- Dutch Bone Marrow Donor Program (The Netherlands)
- Registro Español de Donantes de Médula Ósea (Spain)
- Registo Português de Dadores de Medula Óssea (Portugal)
- Polski Rejestr Dańców Szpiku (Poland)
- Czech Bone Marrow Donor Registry (Czechia)
- Hungarian Bone Marrow Donor Registry (Hungary)
- Registul Naţional al Donatorilor de Celule Stem Hematopoietice (Romania)
- Bulgarian Bone Marrow Registry (Bulgaria)

Asia and the Pacific

- Japan Marrow Donor Program
- The Australian Bone Marrow Donor Registry
- Korean Marrow Donor Program (South Korea)
- DATRI (India)
- China Marrow Donor Program
- Other countries in the Asia-Pacific region, like Singapore, Thailand, Malaysia, and New Zealand, also have their own smaller registries and work through international networks to expand donor searches

Challenges in the Asia-Pacific Region One of the main challenges is the ethnic diversity in the region. For example, patients from South Asia or Pacific Islands often find it difficult to locate a matched donor due to the relatively lower number of donors from these groups in the global registry [4].

Due to the size and geographic isolation of certain countries, logistical challenges arise in transporting stem cells across countries. However, international collaboration among registries and medical centers helps mitigate this problem. In many parts of the Asia-Pacific region, there is still low awareness about stem cell donation, leading to smaller donor pools. Countries like India and China are working to increase recruitment to expand their registries [5].

Middle East and North Africa

- Kök Hücre Bağışçı Kayıt Sistemi (Turkey)
- Saudi Stem Cell Donor Registry (Saudi Arabia)

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- The Egyptian Marrow Donor Registry (Egypt)
- Qatar Bone Marrow and Stem Cell Donor Registry (Qatar)
- Emirates Stem Cell Donor Registry (UAE)
- Kuwait Stem Cell and Bone Marrow Donor Registry (Kuwait)
- Pakistan Bone Marrow Donor Registry (Pakistan)
- Iranian Bone Marrow Donor Registry (Iran)
- Jordanian Bone Marrow Donor Registry (Jordan)

Africa

- The South African Bone Marrow Registry (South Africa)
- The Nigerian Bone Marrow Registry (Nigeria)
- The Kenya Bone Marrow Registry (Kenya)

The Role of Ethnicity and Genetic Diversity in Africa One of the biggest challenges in the stem cell donor search in Africa is the genetic diversity of its populations. African populations have more genetic diversity than any other continent, which means that stem cell matching can be particularly difficult [6]. Many global stem cell registries are predominantly composed of donors from Caucasian or Asian backgrounds, leaving African patients with fewer opportunities to find matches. Patients from African or mixed ethnic backgrounds are often at a disadvantage because fewer African and African-diaspora donors are represented in international registries [7]. However, there are now efforts to address these issues, such as increasing donor diversity [8].

12.2.1.2 Global Collaboration Networks

To increase the chances of finding a match, national registries collaborate on an international level. Several organizations coordinate these global efforts:

- **World Marrow Donor Association (WMDA):** The WMDA is an international body that brings together marrow donor registries, cord blood banks, and transplant centers worldwide. The WMDA facilitates the sharing of donor data across countries to allow for a more comprehensive and efficient search for matching stem cell donors [9].
- **Global stem cell network:** This network connects donor registries around the world, enabling the search for stem cell donors to span multiple countries and continents. It is not an organization itself but rather a collaborative effort among various international registries.

12.2.1.3 International Donor Sharing

The search for an unrelated stem cell donor involves direct data-sharing agreements between national registries. When a patient's transplant team begins searching for a match, they often access multiple registries around the world to maximize the chances of finding a compatible donor. For example:

- Be The Match in the United States has agreements with other major registries like ZKRD, Anthony Nolan, and the Japan Marrow Donor Program, allowing their databases to be searched simultaneously.

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- Similarly, registries in countries like Germany (DKMS), the United Kingdom (Anthony Nolan), and Canada (OneMatch) collaborate through international agreements to expand the donor pool.

12.2.1.4 The Role of Bone Marrow Transplant Centers

Bone Marrow Transplant Centers, which are hospitalsand institutions specializing in stem cell transplants, often serve as the point of contact for patients needing a transplant. The transplant teams at these centers

- Request a search through their national registry (like "Be The Match") for potential donors.
- Work with international registries to widen the search when necessary, especially in cases where a match is hard to find in national pools.

12.2.1.5 International Cord Blood Banks

Some transplants are performed using cord blood, which is collected from the umbilical cord of newborns. The use of cord blood increases the pool of potential donors and can be particularly useful for patients who do not have a well-matched adult donor. Several international cord blood banks, such as the NetCord network, also share data and collaborate on finding matches [10].

12.2.1.6 Coordination and Logistics

Once a donor match is found across international borders, logistics are coordinated by international transplant centers and organ procurement organizations. This involves

- Travel arrangements for stem cell collection, as needed.
- Shipment of stem cells from the donor's country to the recipient's transplant center.
- Legal and ethical considerations, as cross-border transplants involve compliance with various national and international regulations regarding donation, consent, and transportation [11].

12.3 Laboratory Work

12.3.1 HLA Typing

The process of matching involves HLA typing, which identifies specific markers on the surface of white blood cells that are critical for immune recognition. The better the match between the donor's and recipient's HLA types, the lower the risk of transplant complications like GVHD and graft failure (Figure 12.1). It is internationally standard to type 5 HLA loci on a high-resolution level, as the best match is a 10 out of 10. This means that on the loci HLA-A, -B, -C, -DRB1, and -DQB1, both alleles

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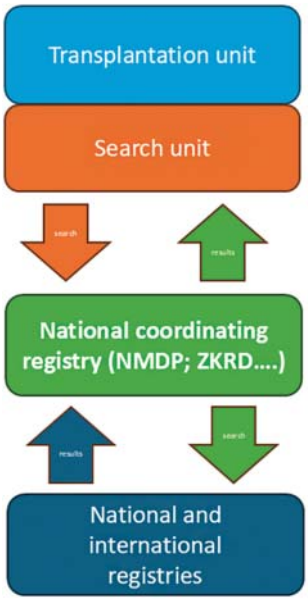

Figure 12.1 Workflow of the donor search in unrelated stemcell transplantation.

of patient and donor are identical [12]. In recent years, a sixth locus, HLA-DPB1, has been added, which is not matched one to one but organized in two groups based on immunogenicity: high and low immunogenicity. The patient and donor should have the same level of immunogenicity to minimize immunological reactions after the transplant [13].

The advent of more sophisticated HLA typing technologies, including high-resolution sequencing of the whole genes and long-range sequencing, has made it possible to achieve more precise donor-recipient typing, for example, without ambiguities even outside the standardized important exons of the HLA genes. Standard without ambiguities are the exons 2 and 3 in HLA class I and exon 2 for HLA class II [14]. The consequence of more precise typing is a more accurate matching. This reduces the risk of complications and improves transplant success rates [15].

However, if no fully matched donor is found, bioinformatic tools are available to predict the acceptance or rejection of the donated stem cells. The tools are based on the knowledge of the structure of the epitopes on the surface of the different alleles and their similarity [16].

Every patient and the chosen donor have to be typed twice to secure the identity of the patient sample and the typing data of the donor stored in the registry's database (Confirmatory Typing) [17].

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12.3.2 Additional Parameters for Donor Selection

Parallelly, the different potential donors are also tested for other parameters, which may influence the transplantation:

- **Blood group compatibility:** ABO blood group compatibility between the donor and recipient is essential, although mismatches can be tolerated to some extent, especially with recent advances in transplantation practices [18].
- **Cytomegalovirus (CMV) status:** Testing for CMV antibodies in both the donor and recipient helps assess the risk of CMV reactivation after transplantation. A CMV-positive donor may be problematic for a CMV-negative recipient due to the risk of infection, or the transplanted "new" immune system immediately has to fight against the preformed virus in the recipient [19].
- **Donor's age and sex:** Younger donors are generally preferred as they tend to provide higher-quality stem cells and have better overall outcomes in transplantations. Donors are typically preferred to be between 18 and 40 years old, although older donors may still be used in some cases [20]. Women may have preformed HLA antibodies due to pregnancies [21].
- **Donor's health status:** Comprehensive health screening of the donor is essential. This includes tests for
  - **Physical exam:** to assess overall fitness and any pre-existing conditions.
  - **Blood tests:** To ensure no underlying infections, anemia, liver disease, or other disorders that could complicate the donation process.
  - **Infectious disease screening:** Testing for HIV, hepatitis B and C, syphilis, tuberculosis, and other infectious diseases [22].
- **Donor's bone marrow function:** If the stem cells are to be harvested from bone marrow (as opposed to peripheral blood), the donor's bone marrow function and health must be evaluated. Good-quality marrow is crucial for successful transplantation [23].
- **Donor's psychological evaluation:** Psychological assessment to ensure the donor fully understands the implications of the donation, the process, and any potential risks [24].
- **Karyotyping and genetic testing (not obligatory):** Genetic testing can help rule out conditions that may predispose the donor to hematologic diseases or other genetic abnormalities. Karyotyping may also be used to check for chromosome abnormalities [25].
- **Donor's medical history:** A thorough review of the donor's medical history, including any previous cancers, blood disorders, or autoimmune diseases that could impact the donation process [26].
- **Donor's current medication profile:** The donor's current medications are assessed to ensure none would interfere with the stem cell collection process or affect the quality of the stem cells [27].
- **Tissue and cell-specific antibody testing:** In certain cases, donor–recipient mismatches can lead to severe graft failure. Assessing the level of donor-specific antibodies may help predict the likelihood of graft failure [28].

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12.4 Clinical Outcomes of Unrelated Stem Cell Transplantation

Clinical outcomes of unrelated stem cell transplanation have improved over the years due to the advancements in matching, GVHD management, and post-transplant care. Survival rates for patients undergoing unrelated SCT depend on factors such as the patient's age, disease type, donor–recipient match, and the occurrence of complications like GVHD [29].

Recent studies indicate that survival rates for patients undergoing unrelated stem cell transplantation are steadily improving, with long-term survival rates ranging from 50% to 80%, depending on the disease being treated. However, complications like chronic GVHD, infections, and graft failure can significantly impact outcomes, requiring ongoing management [30].

12.5 Challenges in Unrelated Stem Cell Transplantation

While unrelated stem cell transplantation has brought remarkable benefits, it remains fraught with challenges.

12.5.1 Donor–Recipient Compatibility

Perfect matching between the donor and recipient remains challenging. Mismatches increase the risk of GVHD and graft failure, affecting the overall success of the transplant. Despite advances in matching technologies, the issue of finding a perfectly compatible donor, particularly for patients from ethnic minorities, remains a concern [31].

12.5.2 GVHD Prevention and Treatment

GVHD remains one of the most significant challenges in unrelated stem cell transplantation, especially when no compatible donor was found. Recent advancements in immunosuppressive therapy have improved outcomes by better controlling the recipient's immune response and minimizing GVHD. Drugs like tacrolimus and methotrexate, when used in combination, help prevent GVHD, while newer approaches like T-cell depletion and mesenchymal stem cell infusion are being explored to further reduce the incidence of GVHD without compromising the patient's immune function [32].

12.5.3 Economic and Ethical Considerations

The cost of SCT, especially when using unrelated donors, is significant. Expenses related to donor recruitment, matching, stem cell collection, and post-transplant care can be prohibitive for some patients, particularly in developing countries. Ethical issues also arise regarding donor compensation, the privacy of genetic information, and equitable access to SCT across different socioeconomic groups [33].

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12.5.4 Case Studies

Case studies highlight the success and challenges of unrelated SCT in clinical practice. For example, a 2018 study on patients with acute leukemia found that survival rates were higher in patients who received stem cells from well-matched unrelated donors compared to those who received haploidentical-related transplants [34].

12.6 Future Directions and Emerging Technologies

The future of unrelated stem cell transplantation is promising, with several emerging technologies that could revolutionize the field.

Gene editing technologies, particularly CRISPR-Cas9, are being explored to modify stem cells before transplantation. This could help correct genetic defects in patients with inherited disorders or enhance the graft-versus-leukemia effect while minimizing the risk of GVHD and curing additional diseases of the patient like HIV [35].

The development of engineered stem cells and the use of bioengineering to enhance graft survival and reduce complications is another exciting avenue. In addition, stem cell banks that store cord blood and peripheral blood stem cells could improve accessibility and reduce waiting times for patients in need of a transplant [36].

The future of unrelated stem cell transplantation may also lie in personalized medicine, where genetic profiling is used to tailor treatment protocols based on individual patient characteristics. This could lead to better outcomes with fewer side effects [37].

12.7 Conclusion

Unrelated stem cell transplantation has significantly advanced the treatment of many life-threatening diseases, providing a second chance for patients who would otherwise have limited treatment options. Despite challenges related to donor matching, immunological complications, and the high cost of treatment, ongoing research and technological innovations are improving success rates and making this therapy more accessible.

The future of this therapy is bright, with emerging technologies such as gene editing, personalized medicine, and advances in bioengineering offering promising solutions to current challenges. Continued collaboration at the global level, the expansion of donor registries, and the development of new therapeutic strategies will ensure that more patients benefit from this life-saving treatment.

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13

Chimerism Testing in Stem Cell Transplantation

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13.1 Introduction

A chimera is a monster in Greek mythology having a lion's head, a goat's body, and a serpent's tail. Never seen and classified by Lamarck in 1794 [1], the chimera finally appeared in scientific literature in the twentieth century. A biological chimera is an individual whose body is composed of cells of diverse genetic constitutions; either from the same individual, for example, due to the fusion of two different zygotes within a single embryo [2], or from different individuals. The term chimerism was introduced into the field of transplantation in 1956 [3] and is nowadays most commonly used to describe the stages of hematopoietic chimerism after hematopoietic stem cell transplantation (HSCT). The state of complete chimerism (CC) is diagnosed when only donor-derived blood cells are found in the peripheral blood (pB) or bone marrow (BM) of the patient. Mixed chimerism (MC) means that the patient shows both recipient and donor cells in pB or BM. Chimerism analysis is used after HSCT to monitor the engraftment or detect the recurrence of recipient-derived cells and therefore a potential relapse of leukemia. A hematopoietic chimerism can also be established after solid organ transplantation (SOT) when immunocompetent cells transferred together with the graft proliferate in the immunosuppressed recipient. The risk to develop an acute graft-versus-host disease (GvHD) increases with the amount of lymphocytes transferred with the transplant. In kidney transplantation, only a few GvHD cases are reported in the literature, whereas for the liver, which is rich in lymphoid tissue, the GvHD incidence is 0.1–2%. GvHD after liver transplantation has a mortality rate of above 60% [4]. Chimerism analysis is a helpful tool to support the diagnosis of GvHD after SOT. Chimerism can also be acquired naturally, which can have a diagnostic and clinical impact. A hematopoietic microchimerism (chimeric population constitutes <1%) can occur due to a bidirectional transplacental transfer of blood cells during pregnancies. Materno-fetal chimerism can persist for many years;

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feto-maternal chimerism can persist for decades or even lifelong in the mother [5, 6]. A similar microchimerism occurs between monochorionic twins, which is present in 8% of twin pairs and 21% of triplet pairs [7]. There are also other rare forms of chimerism like tetragametic chimerism. Feto-maternal microchimerism seems to be associated with the development of autoimmune diseases like systemic sclerosis, Sjogren's syndrome, and systemic lupus erythematosus, which clinically resemble GvHD [6]. The positive aspect is that feto-maternal chimerism seems to facilitate haploidentical HSCT with undepleted (T-cell) grafts [8]. Additionally, haploidentical HSCTs with microchimeric, noninherited maternal antigen (NIMA) mismatched donors have a low risk of GvHD [9]. Microchimerism may produce long-lasting tolerance and seems to be an important factor in the induction of NIMA-specific tolerance. Naturally occurring microchimerism can be much more pronounced in immunodeficient individuals. For example, infants with severe combined immunodeficiency and engrafted maternal stem cells can develop GvHD symptoms. If these patients undergo HSCT, they have a decreased risk of rejecting a maternal graft but a higher risk of developing GvHD in the presence of maternal antigens [10].

The basic principle of chimerism testing is to identify genomic markers/or alleles in which the donor and recipient differ. A genetic marker that is present in the donor but not in the recipient is informative for the donor and vice versa, a genetic marker that is present in the recipient but not in the donor is informative for the recipient. This information, including information on homozygosity or heterozygosity of the informative marker, is used to quantify the recipient/donor or donor/recipient ratio in the follow-up sample. Due to this principle, chimerism testing cannot differentiate between malignant and benign cells. This can only be defined with minimal residual disease (MRD) diagnostics, which uses disease-specific genetic markers. Detecting also benign cells after HSCT with chimerism testing can also be advantageous. These cells can originate from the recurrence of recipient hematopoiesis or reactivation of virus-specific T cells, which can be an indication of over-immunosuppression.

13.2 Methods for Molecular Genetic Chimerism Testing

The first DNA-based chimerism analysis that was commonly used after HSCT was restriction fragment length polymorphism (RFLP) analysis in the 1990s. In this method, genomic DNA of the recipient and donor is digested with restriction enzymes. Polymorphism within the recognition sites of the enzymes results in different DNA fragments for the recipient and donor. For a quantification of the fragments, the Southern blot method can be used. Today, RFLP has been nearly completely replaced by other methods. A survey in November 2019 showed that the short tandem repeats (STRs) followed by real-time quantitative PCR (qPCR) and next-generation sequencing (NGS) are the most commonly used methods [11]. Digital droplet PCR (ddPCR) is another commonly used method for chimerism testing with available commercial kits.

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13.2.1 Short Tandem Repeat

Microsatellites or STR markers are polymorphic gene loci containing repeated nucleotide sequences typically with 2–10 nucleotides per repeated unit (e.g., (AGGC)n, a tetranucleotide repeated n-times). The alleles of an STR have different number of repeats. For STR analysis, different STR loci are amplified in a multiplex PCR using different fluorescence-labeled PCR primers. The PCR products are then separated by length using capillary electrophoresis, whereas the STR loci can be differentiated by the length of the PCR product and the fluorescence dye. The alleles of a specific locus are identified by length (number of repeats). An individual will normally be homo- or heterozygous for an STR allele in a marker system, resulting in one or two allelic peaks in an electropherogram, respectively. The post-transplant sample can have up to four allelic peaks. The percentage of the chimerism is calculated with the area under the peaks of the electropherogram, which correlates with the amount of templates of the STR allele. A peak is informative for the donor when it is only present in the donor (and vice versa for the recipient). A schematic STR analysis is shown in Figure 13.1. The formula for the calculation depends on the STR marker constellation (homo- and heterozygosity, alleles shared or not shared by donor and recipient). When the recipient and donor share no allele, the following formula can be used:

$$\%\ \text{chimerism for donor engraftment} = \frac{(C + D)}{(A + B) + (C + D)} \times 100$$

(C and D are the peak areas of donor alleles, A and B are the peak areas of recipient alleles).

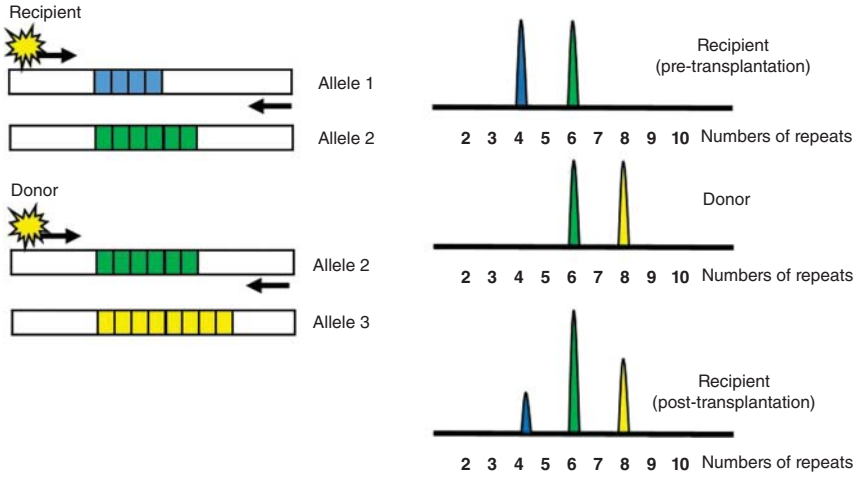

Figure 13.1 A hypothetic STR marker in three different allelic manifestations, the blue allele has four repeats, the green allele has six repeats, and yellow allele has eight repeats. The arrows represent the PCR primers, whereas the forward primer is labeled with fluorescence dye. On the right, the electrophoretograms are depicted. The blue allele is informative for the recipient; the yellow allele is informative for the donor. The area of the yellow peak is twice as much as the area of the blue peak; therefore, one-third of the cells of the post-transplant sample are recipient cells.

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This STR marker constellation is called fully informative, and it is recommended, where possible, to use this constellation for calculation of % chimerism [12].

In case of the constellation of Figure 13.1:

$$\%\ \text{chimerism for donor engraftment} = \frac{\text{allele 3}}{\text{allele 1} + \text{allele 3}} \times 100$$

Allele 3 is donor-specific, allele 1 is recipient-specific, and allele 2 is shared by donor and recipient (shared allele).

Despite the informativity, a marker can sometimes not be used due to the presence of stutter peaks. The length of the stutter PCR products is typically $n - 1$ of the allelic PCR products. Stutter peaks result most likely from slipped-strand mispairing during amplification [13]. Sensitive STR methods can detect at maximum a minority just below 1%.

13.2.2 Quantitative Real-Time PCR

The primers of the qPCR are usually targeting biallelic single-nucleotide polymorphism (SNP) or insertion/deletion (InDel) polymorphism. InDel markers have a low risk of unspecific amplification of the untargeted allele. The amount of templates produced during the qPCR is measured with TaqMan probes. These probes are specific for the template, and during the elongation step of the PCR cycle, the 5′ to 3′ exonuclease activity of the polymerase causes the hydrolyzation of the bound probes, and in consequence releasing the fluorophore from its quencher. The increase in the fluorescence is then measured after every PCR cycle. The reactivity of a DNA in qPCR is represented by the cycle threshold (Ct) value. The Ct value represents the cycle number at which a sample's reaction crosses a fluorescence threshold. The threshold must be within the exponential phase of the PCR. A difference of 1 between the Ct values of two amplification curves (delta Ct or dCt of 1) indicates that the DNA with the lower Ct value is twice as highly concentrated as the DNA with the higher Ct value.

Prior to the quantification, a screening for informative markers has to be performed (the recipient and donor are typed positive or negative for the genetic markers). The quantification step entails qPCRs with the DNA of the follow-up sample and the DNA of the patient before transplantation, where the latter represents the 100% reference sample. For each sample, two qPCRs are performed, one with primers and probes for the informative marker and the other with primers and probes for a reference marker (nonpolymorphic gene). Differences in the DNA quantity and quality between the reference sample and the follow-up sample are normalized with delta Ct (dCt) values of the reference gene and the informative markers (Figure 13.2). The % chimerism is calculated as follows:

$$\begin{aligned}
\Delta\text{Ct1} &= \text{Ct (informative marker/post HSCT sample)} \\
&\quad - \text{Ct (reference marker/post HSCT sample)} \\
\Delta\text{Ct2} &= \text{Ct (informative marker/pre HSCT sample)} \\
&\quad - \text{Ct (reference marker/pre HSCT sample)} \\
\Delta\Delta\text{Ct} &= \Delta\text{Ct1} - \Delta\text{Ct2};\ \%\text{Chimerism} = 2^{-\Delta\Delta\text{Ct}} \times 100
\end{aligned}$$

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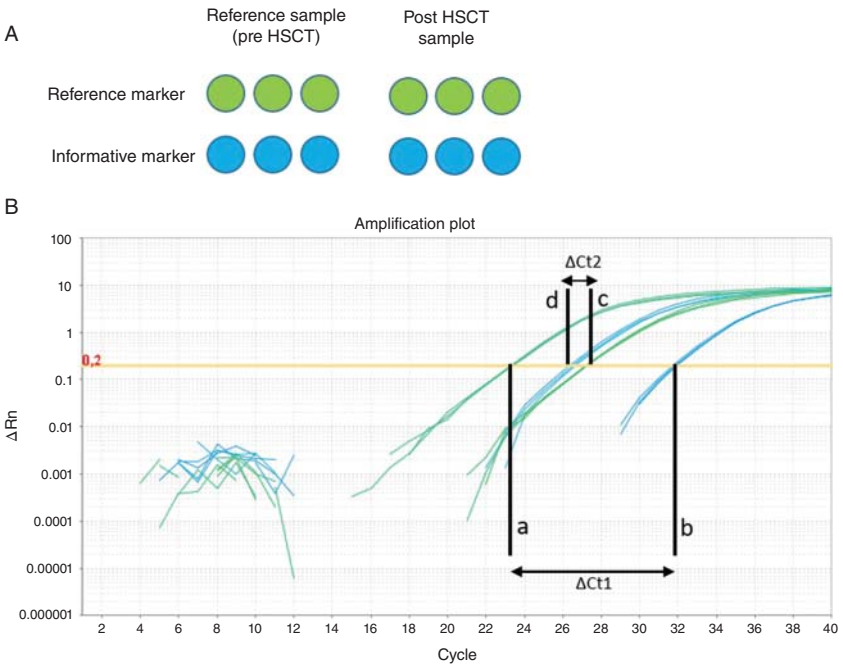

Figure 13.2 (A) Pipetting scheme for a qPCR assay. In order to increase the accuracy and calculate a standard deviation, every DNA/marker combination is pipetted in triplets. (B) qPCR plot of a chimerism assay for one marker. Threshold for the slopes was set to 0.2 ΔRn (normalized fluorescence). Slopes of the PCR of the reference marker with post sample (a), informative marker with post sample (b), reference marker with reference sample (c), and informative marker with reference sample (d) are shown. DNA input was 100 ng for the post (follow-up) sample and 10 ng for the reference sample. The follow-up sample had a recipient chimerism of 0.17%.

13.2.3 Digital Droplet PCR

ddPCR is based on a water–oil emulsion, whereby each droplet of the emulsion forms a separate reaction sphere for the PCR. The applied amount of DNA is adjusted to result in droplets containing most of the time zero, one, or two and rarely more than two copies of the targeted template. The master mixes are composed of the primers for the amplification of a biallelic marker (usually InDel marker) and a reference gene as well as the two corresponding TaqMan probes. The droplets are thermally cycled, and after the last cycle, an end-point measurement of the fluorescence is performed. The droplets will either have the fluorescence signal for the informative marker (e.g., HEX), the reference gene (e.g., FAM), both markers, or no signal (see Figure 13.3). With an initial genotyping of donor and recipient (pre-transplant) samples, informative markers and their copy numbers (homo- or heterozygosity) are identified. In a second step, ddPCRs with the DNA of the follow-up sample and one or more discriminative markers are performed. The % chimerism is calculated using Poisson statistics and the information on the copy numbers of the markers.

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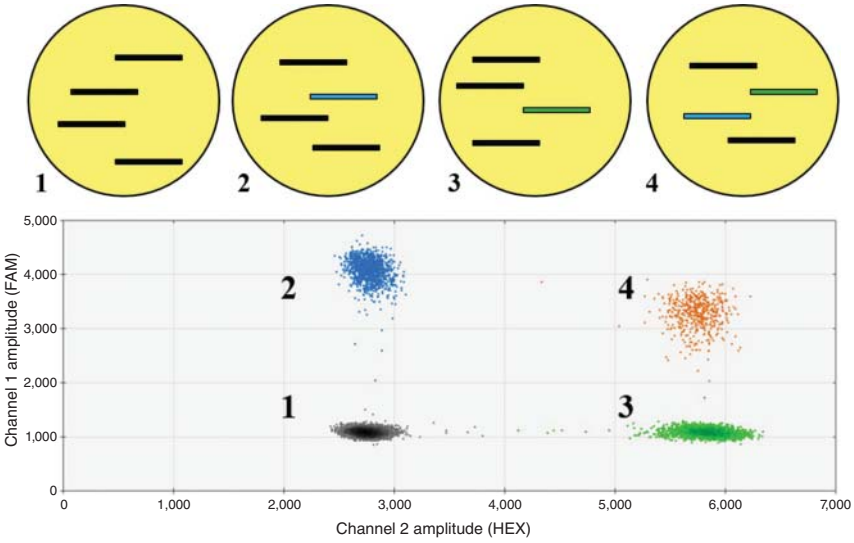

Figure 13.3 Droplet 1 contains templates neither of the marker nor of the control gene. Droplet 2 contains one or more templates of the marker (blue line). Droplet 3 contains one or more templates of the control gene (green line). Droplet 4 contains templates of the marker as well of the control gene. The fluorescence plot below shows the counts for these droplets.

13.2.4 Next-Generation Sequencing

For NGS chimerism testing, 24–202 different biallelic markers are amplified in a multiplex PCR (in one or two PCR reactions). The 5′-ends of the PCR primers are already tagged with the binding sites for the indexing, which simplifies and shortens the library preparation. With the massive parallel sequencing approach of NGS platforms, the genetic markers are sequenced several hundred times (deep sequencing). The sensitivity of the NGS chimerism is mainly defined by the read depth (number of reads of an allele or coverage of a polymorphic position), together with the amount of DNA used for amplification. Other factors influencing the sensitivity are the signal-to-noise ratio and the number of informative markers used for the % chimerism calculation. A marker is informative when one of the alleles is unique for the recipient or the donor (a marker is noninformative when the donor and the recipient are both homozygous for the same allele or both are heterozygous). The % chimerism is calculated by comparing the ratio of allele 1 and allele 2 reads of a marker (see Figure 13.4). The final % chimerism is usually reported as the average value of all informative markers measured in a particular donor/recipient setting. A factor limiting the sensitivity is the sequencing noise (error rate of NGS > 0.1–1%), which can occur due to miscalled bases (detection device/algorithm), DNA damage, and DNA polymerase errors [14]. A very common mechanism leading to baseline noise during NGS seems to be cytosine deamination, resulting in C:G > T:A mutations [15]. The sensitivity can be increased far below the noise limit using InDel markers with multiple Insertion/deletions, for which

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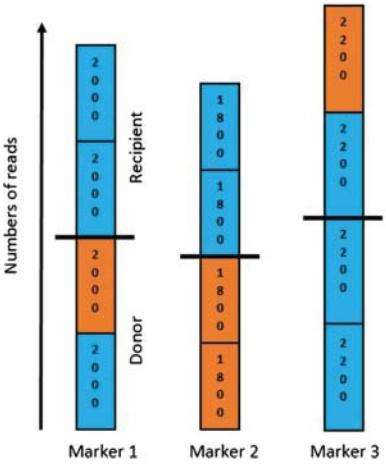

	Marker 1	Marker 2	Marker 3
Recipient	short/short	short/short	short/long
Donor	short/long	long/long	short/short
Nos. reads	8000	7200	8800
%short reads	75%	50%	25%
%long reads	25%	50%	75%

Figure 13.4 Idealized example of NGS chimerism assay with InDel markers. Alleles with a deletion result in short reads (blue) and alleles with an insertion result in long reads (orange). The patient has a 50% recipient chimerism. The upper half of the columns represents the number of reads from recipient-derived alleles, whereas the lower half of the columns represents the number of reads from donor-derived alleles. The number of maternal and paternal alleles is given in the subdivided recipient and donor columns. The Table depicted in this Figure shows the typing of the recipient and donor, and total number of reads and percentage of short and long reads for these markers.

the analysis software discriminates short from long reads and thereby eliminates the impact of single-nucleotide errors. SNP markers are more prone to sequencing noise but the noise can be subtracted out using a large number of informative markers to calculate the % chimerism.

13.2.5 Comparison of the Methods

The methods for chimerism testing are compared in Table 13.1 with the aid of the following parameters:

Discrimination: Chance to find an informative marker. Forensic DNA profiling utilizes also the STR method. In principle, this method can distinguish all possible recipient/donor constellations (except for monozygotic twins). For the other methods, the discrimination power depends on the number of available genetic markers.

Quantity of DNA: Quantity of DNA from follow-up samples needed for quantification. The amount of DNA applied in qPCR assays can be individually adapted. The DNA amount can range from 10 up to 100 ng per PCR reaction, whereas the detection limit then consequently ranges from 1 to 0.1%, respectively. In case of aplastic patients, a sufficient DNA yield can be problematic. Another disadvantage of the qPCR is that for every quantification DNA of a pretransplant sample is needed (~10 ng per PCR reaction).

Sensitivity: Detection Limit of the Minority

Time to relapse: Time from first detection of MC to diagnosis of hematological relapse. The prediction time depends on the detection limit of the assay. The average prediction time of STR and qPCR is 38 and 120 days, respectively [18].

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Table 13.1 Comparison of methods for molecular genetic chimerism testing.

	STR	qPCR	ddPCR	NGS
Discrimination	+++	+	+	++
Quantity of DNA	<5 ng	>150 ng	150 ng	60–100 ng
Sensitivity	1–5%	0.01–0.1%	0.05–0.1%	0.05–0.5%
Time to relapse	+	+++	++(+)	++(+)
Accuracy	++	+(+)	++	++
Time to result	15 h	<3 h	<3 h	>24 h
Throughput	++	+(+)	++	+++

Source: Adapted from Picard et al. [16] and Heinold et al. [17].

Accuracy: Usually, the highly sensitive qPCR and ddPCR methods use recipient-specific markers to calculate the % chimerism. However, with a very dominant proportion of donor cells, these assays lose accuracy. In consequence, such samples should then be analyzed in qPCR and ddPCR assays with donor-specific markers. NGS assays use informative markers of recipient and donor to calculate the % chimerism. When measuring samples with a dominant majority (>90%) with NGS, only informative markers for the minority should be considered for the calculation of the % chimerism.

Time to result: A turnaround time of less than five working days for routine and less than three working days for urgent requests is recommended [12]. With the isolated DNA at hand and a preexisting typing (identification of informative markers), the qPCR is the most rapid method, with approximately 100 minutes until reporting a result. The runtime of an NGS sequencing run depends on the capacity of the cartridge/flowcell (output in gigabase, Gb) and the number of cycles. A run with a low or medium output will usually take less than 17 hours, a high output 24 hours. The time needed for collecting enough samples to utilize the capacity of the cartridge/flowcell should also be considered for the turnaround time.

Throughput: The number of capillaries (1–96) for the electrophoresis is the limiting factor for STR. In case of the qPCR, the throughput can be increased using a 384-well block instead of a 96-well block, which, however, also increases the complexity of the pipetting. Aiming to reach a sensitivity of 0.1% with NGS, a low-output run (1.2 Gb) can be loaded with 8–11 samples and a high-output run (6.6–7.5 Gb) with up to 44–68 samples (numbers differ between the vendors).

13.3 Sensitivity of Molecular Genetic Chimerism Testing

The methods for molecular genetic chimerism testing are based on the principle that the detection of one gene copy of a heterozygous gene or two gene copies of a homozygous gene is equivalent to the detection of one cell.

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Therefore, the sensitivity limit is always directly dependent on the amount of DNA used in the assays. A human cell contains approximately 6 pg of DNA. With 60 ng of DNA corresponding to approximately the amount of 10,000 cell equivalents, 1 in 10,000 cells can be detected in this amount of DNA. If 60 ng of DNA is used, the maximum sensitivity limit for the detection of a heterozygous marker is 0.01% and for a homozygous marker 0.005%. To increase the probability that a DNA template of the minority is present in the assay, a safety factor of 10 should be applied (10 out of 10,000 cells). The theoretical sensitivity limit in this case would therefore be for a homozygous marker approximately 0.1% and for a heterozygous marker 0.05%.

DNA can be degraded, contaminated with PCR inhibitors, or contain substances with similar physical/chemical properties like DNA. Such DNA will show, for example, in photometric measurement of nucleic acids, an apparently good quantity or quality but will not be amplified very well in a PCR. This can have a direct impact on the sensitivity of a PCR. For example, qPCR with a Ct value of 30 instead of 24 for the reference marker of the follow-up sample will have a detection limit of 0.34% instead of 0.01% [17]. Laboratories are often confronted with low-quality/quantity DNA from aplastic patients, and the clinicians may urgently ask for an immediate result to diagnose, for example, engraftment failure. There should be no problem in testing these DNAs as long as a substantial number of cells of the minority are detected with the assay. But if the assay does not detect cells of the minority, parameters correlated with the amplification capability of the DNA have to be checked and in that case the sensitivity level has to be adjusted [17]. These parameters can be the Ct value (qPCR) of the reference gene, total number of positive droplets (ddPCR) of a marker, or the total read depth of a marker (NGS).

13.4 Choice of Markers for Quantification

One of the most pervasive genetic characteristics of cancer cells involves chromosomal gains and losses, which are nearly always associated with aneuploidy [19]. These differences in copy numbers of genetic markers between leukemic cells and healthy blood cells can have important implications for chimerism testing. A duplication of a chromosomal region carrying an informative marker used for chimerism testing will result in an overestimation of the recipient-derived % chimerism. Even more impactful for chimerism testing is a loss of heterozygosity (LOH), which typically comprises the loss of large chromosomal regions associated with tumor suppressor genes. An informative marker affected by an LOH will not be able to detect leukemic cells. This has two implications for the choice of markers. Firstly, markers should be chosen with the help of the karyotype of the leukemic cells; markers that are located on the same chromosome (arm) as a known deletion or duplication should be avoided. Secondly, at minimum two markers, which are located on different chromosomes, should be used to calculate the % chimerism. The latter is important, because during the progression of a relapse, the leukemic cell can acquire new LOHs in the regions of an informative marker [20].

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In general, more than one marker should be assayed as it allows the comparison of results or reporting of a mean. For STR assays, the use of three or more different markers, where possible, is recommended [12]. For some assays using biallelic markers, it can be problematic to find more than two informative markers, especially in case of sibling transplantation. For sibling transplantation, at least 40 biallelic markers are needed to allow reliable chimerism monitoring in over 99% of the transplants [21]. With a limited set of markers, it is possible that no informative markers can be identified. The accuracy of qPCR assays should be increased by setting up the reactions in triplicates.

13.5 Chimerism Testing for Nonmalignant Disorders

A complete donor chimerism for nonmalignant disorders (e.g., primary immunodeficiencies, thalassemia, sickle cell disease) is usually not needed and the proportion of healthy donor cells has just to be sufficient to keep the patient free of severe symptoms [22]. In the case of thalassemia, patients with a recipient % chimerism of more than 30% have an increased risk of graft rejection and of becoming transfusion-dependent again. In sickle cell disease, the proportion of donor cells needed for an effective treatment depends on whether the donor has the sickle cell trait (HbAS) or not. For transplantations with a heterozygous HbAS donor, 30–40% donor cells are required, whereas for a homozygous healthy donor only 10% of donor cells are required [22]. Therefore, highly sensitive chimerism testing is not needed for these diseases. However, for metabolic neurodegenerative disorders a prompt and high-percentage donor engraftment is required to stop neurodegeneration as early as possible [23]. Although patients with inherited BM disorders have a predisposition to myeloid malignancies, less effort needs to be put into the selection and number of markers considering LOH or other chromosomal abnormalities.

13.6 Reporting Chimerism

The percentage ratio between the number of donor and recipient cells (% chimerism) can be reported as the fraction of donor or recipient cells. Reporting the % donor chimerism (amount of donor cells divided by the total amount of cells and multiplied by 100), a 100% value will represent a full donor chimerism. The other way around, reporting % recipient chimerism, a 0% value will represent a full donor chimerism. In order to avoid any confusion, the report has to express unequivocally which fraction is meant. A chimerism report should have, compared to other H&I testing, several additional information:

Donor identity: The donor, which was used to identify the informative marker, has to be stated on the report. A mix-up of donors can lead to completely wrong or, in the best case, inconclusive results.

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Date of transplantation: The date and number of transplantations (first, second, or third) has to be given. The latter is important because a second (or third) transplantation can have been performed without notifying the laboratory.

Sample source: The specimen used for testing has to be mentioned (e.g., pB, BM, or purified cells). BM samples differ from PB samples as they can contain stroma cells and in case of a relapse the % recipient chimerism can be more than a magnitude higher compared to pB (see below). Cell line-specific chimerism results should include information about the purity of the specific cells.

Detection limit: At least in case of a CC, the detection limit has to be written on the report.

Informative markers: The informative markers used for the quantification and their chromosomal location should be stated. PCR's efficiency and other factors can differ between markers; a change in markers during a time course can therefore complicate the interpretation of the kinetics of % chimerism. In case of chromosomal aberrations, the chromosomal location of the markers can be helpful in interpreting inconclusive results.

13.7 Time Course of Chimerism Analyses

The dynamic of an increase or decrease of the % chimerism is most important for the early prognosis of relapse or therapeutical interventions (e.g., adjusting immunosuppression). Clinically important is not the highly accurate determination of the % chimerism at a single time, but the accurate determination of increments or decrements and their dynamics [24–26]. In the following, the main characteristic categories of chimerism dynamics are summarized.

Category 1: Continuous complete donor chimerism starting with day 28 after stem cell transplantation.

Category 2: Initially low % recipient chimerism (below 5%), which steadily decreases. Categories 1 and 2 have a much better disease-free survival compared to patients with an increase of % recipient chimerism in their time course [24, 27]. A continuous decline in the proportion of recipient cells may indicate a graft-versus-leukemia (GvL) effect [28].

Category 3: Some patients show, after achieving a complete donor chimerism, a one-time slight increase in the % recipient chimerism (usually less than 1%) with an immediate decline. This type of dynamic is often associated with the release of cell-free DNA (cfDNA) and has no malignant background (own data). cfDNA can originate from surgery, injuries, necrosis, infarction, inflammation, and GvHD [29, 44]. Patients after HSCT have an average of 469 ng/ml (range: 50–10,700 ng/ml) of cfDNA. cfDNA is fragmented (on average, 80–200 base pairs long) and quickly degraded [29]. Highly sensitive chimerism assays that amplify small fragments can detect this cfDNA. Figure 13.5a shows the course of a patient who underwent surgical treatment (excision of necrotic tissue) at the same time as the short spike in recipient chimerism occurred.

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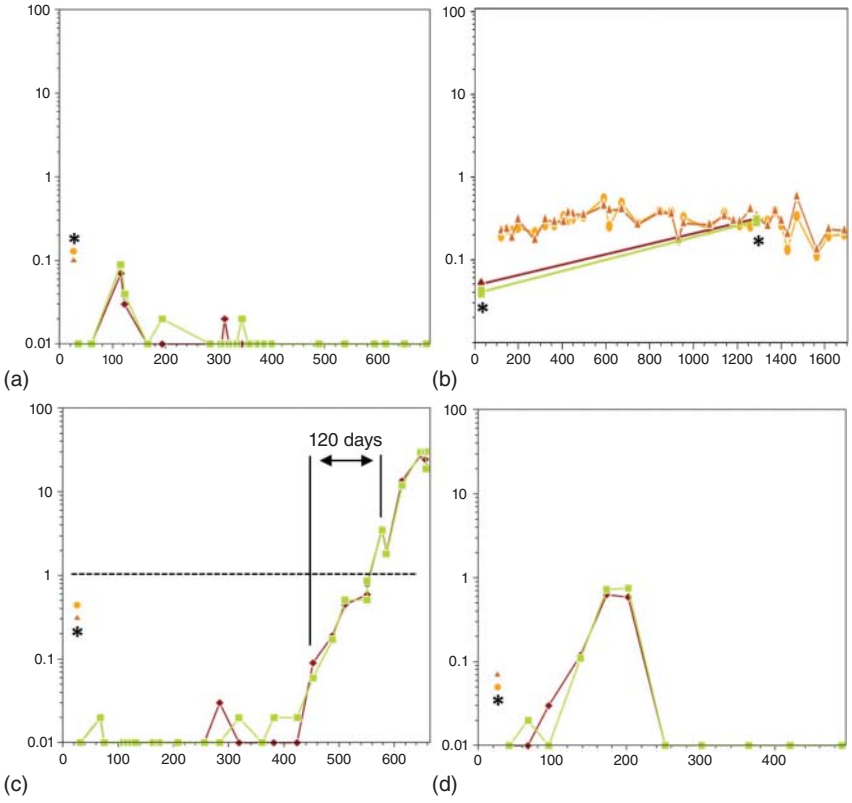

Figure 13.5 X-axis, days after transplantation. Y-axis, percentage recipient chimerism. Each sample was measured with two different markers. BM samples are marked with a *; all other samples are pB. Time courses of chimerism monitoring for a patient who underwent surgical treatment (a), a patient with benign autologous hematopoiesis (b), a patient with relapse (c) and a patient with an increase in viral load (d). Images from the analysis software KMRengine®, GenDx.

Category 4: Continuous and only in a weakly manner fluctuating or slowly rising or falling proportion of recipient cells. This proportion is often due to benign autologous hematopoiesis (Figure 13.5b).

Category 5: Two or more consecutive increases in % recipient chimerism. If two increases in chimerism (recipient cell proportion) of more than 0.1% follow one another, the probability of a relapse is very high (HR: 9.4, 95%; CI: 3.8–23; $P < 0.0001$) [25]. The faster and stronger the increase occurs (high dynamic), the higher the probability of a recurrence [26]. Even at very low % recipient chimerism, a doubling from 0.1% to 0.2% within two weeks can be a strong indication of a relapse. This means that with the help of highly sensitive methods, recurrences can be detected very early. Figure 13.5c shows the course of a patient with a relapse. Compared to a method with a detection limit of only 1% (dashed line), the highly sensitive qPCR was able to predict the recurrence approximately 120 days earlier in this case. In addition to a recurrence, over-immunosuppression

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and viral infections can also lead to a repeated increase in % recipient chimerism in the early stage after transplantation. The increase caused by residual stem cells or virus-specific lymphocytes is usually less dynamic than in the case of a relapse [26]. Figure 13.5d shows the course of a patient with an increase in viral load (CMV and EBV). After the immunosuppression was reduced, the % recipient chimerism dropped again. If there is a consecutive increase, a suspected recurrence must be clarified by further diagnostics.

13.8 Advantages and Disadvantages of Highly Sensitive Chimerism Testing

The detection of microchimerism can have several advantages. A repeated increase of the recipient % chimerism, even in the lowest percentage range, is associated with a hematopoietic relapse. This very early detection of small quantities of recipient cells makes it possible to predict a relapse more than three months earlier than with the less sensitive STR method [18] (see also Figure 13.4c). In the first year after transplantation, the detection of microchimerism can be helpful in tapering immunosuppression. Another advantage of highly sensitive chimerism testing is that BM samples have to be taken less frequently. With less sensitive methods, a BM sample is required, as in some cases the proportion of leukemic cells in BM can be 10 times and more higher than in pB. This "early detection advantage" of BM with the less sensitive STR method can also be achieved in pB using methods with a 10–20 times higher sensitivity. A disadvantage of the highly sensitive methods is that also biological background (e.g., contamination with recipient tissue, cfDNA) can be detected and misinterpreted.

13.9 Cell Sources for Identifying Recipient- and Donor-Specific Markers

For the identification of recipient- and donor-specific markers, the DNA should come from a pretransplantation sample. If a pretransplant sample of the recipient is not available, alternative cell sources such as buccal swab, hair follicles, or fingernails can be used. Small amounts of donor-derived cells in the typing sample may not interfere with positive or negative typing of a marker for a particular individual, but fingernails and buccal swabs may contain a significant proportion of donor cells (70% and more) [30, 31]. Such a high proportion of donor cells in the recipient's typing sample will lead to invalid or false typing results. Especially an oral mucositis leads to the infiltration of the mucosa with donor-derived leucocytes, increasing the percentage of donor cells in a buccal swab significantly. Hair follicle cells are almost free of donor-derived cells and contain less than one donor cell among a few thousand recipient cells and are therefore probably the best source for typing [31]. If no DNA of the donor is available, a post-transplant sample of the

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recipient can be applied for the typing of the donor. This should only be done when a presumably full donor chimerism is supported by other findings (e.g., negative MRD, blast count, FISH chimerism) and the typing result is seriously checked for potential recipient signals.

13.10 Cell Sources for Chimerism Monitoring

The most common sources for chimerism testing are pB and BM. Drawing BM is, besides being unpleasant or even painful for the patient, associated with risks like bleeding and infection. These risks have to be balanced with the benefit of increased numbers of CD34-positive cells and potentially leukemic cells within the BM sample. A problem with BM is that it contains a certain amount of stromal cells from the recipient [32]. The proportion of "contamination" depends on the sampling technique and the condition of the BM. For example, BM aspirates of patients with BM fibrosis are often hypocellular or even acellular and in the worst case unsuccessful (punctio sicca), which influences the proportion of stroma cells within the sample. According to the experience of our laboratory, BM can contain between 0.01% and 1% (on average, between 0.05% and 0.5%) stroma cells. Using highly sensitive methods, these fluctuations in the proportion of stroma cells in the BM can make it difficult or impossible to detect an increase in leukemic cells. In case of extramedullary relapses, any type of tissue biopsies or fluids (e.g., liquor cerebrospinalis) may be sent to the laboratory. Specific cell lines can be purified and subsequently examined in a cell lineage-specific chimerism test. Progenitor cells (CD34+), T cells (CD3+), B cells (CD19+), myeloid cells (CD33+, CD15+), and NK cells (CD56+) are commonly purified (34, 35). Based on the leukemia-associated immunophenotype (LAIP), specific cell surface markers can be selected to purify pB or BM in order to increase the sensitivity of chimerism testing for the malignant cell line. Since 70–80% of AML have a CD34-positive LAIP, CD34 lineage-specific chimerism is frequently analyzed. By purifying progenitor cells, the sensitivity of STR can be increased to the level of qPCR [33]. To monitor engraftment, chimerism testing in the NK cell population can be performed within the first 28 days. In this early phase, patients with an NK cell donor engraftment below 70% have an increased risk of graft rejection. Similarly, a progressive loss of donor T-cell chimerism is associated with graft rejection and relapse [34].

13.11 Intervals for Chimerism Testing

After myeloablative HSCTs, most relapses (incase of acute myeloid leukemia [AML], acute lymphocytic leukemia [ALL], myelodysplastic syndrome [MDS]) occur within the first three years after transplantation. Between three and six years after HSCT, only a few relapses occur, and after more than six years relapses can occur very rarely [35]. The heterogeneity of the underlying disease, the heterogeneity within a disease entity (e.g., AML), and other transplant-related parameters (e.g., reduced

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Table 13.2 Proposed time intervals for chimerism testing.

Time after transplantation	Intervals
Less than 6 months	Every 2–3 weeks
Between 6 months and 3 years	Monthly
Between 3 and 6 years	Quarterly
More than 6 years	Biannually

intensity conditioning or T-cell-depleted transplants) make it difficult to predict the probability and the most likely time frame for a relapse [36]. In order not to miss the time of the occurrence of a relapse and thus the possibility of early treatment (e.g., pre-emptive DLI administration), time intervals for chimerism testing should be established. This should be always done in concordance with national or international guidelines and considering the detection limit of the chimerism assay. The time intervals are shown in Table 13.2.

13.12 Chimerism-Related Diagnostics

13.12.1 Inside HLA Chimerism (HLA Loss Diagnostic)

Leukemic cells of patients with an HLA mismatch in GvH/GvL direction can escape the pressure of the transplanted immune system by losing the expression of the mismatched HLA allele. A common escape mechanism is the deletion of the mismatched haplotype (deletions of a single HLA locus or a complete HLA class I or II loss are also reported). The LOH of HLA loci compromises usually the duplication of matched HLA loci (uniparental disomy). The incidence of HLA loss was around 30% in relapsed patients with myeloid malignancies after haploidentical HSCT [37, 38], but this number may be different for patients with post-transplant cyclophosphamide protocols. The LOH of HLA loci has serious implications for a patient's therapy. The administration of a donor lymphocyte infusion would mainly cause GvHD and less desired GvL effect. In case of a second transplantation, the new donor should not carry the same HLA haplotype as the one affected by LOH.

The only commercial kit available for diagnosing HLA loss is based on qPCR. This kit can identify informative markers (recipient-specific SNPs within HLA alleles) in approximately 70% of haploidentical HSCT. Due to cross-reactivity of HLA alleles in the qPCR, this assay has a low sensitivity and can therefore only be applied for patients with an outside-HLA chimerism ("normal chimerism") above 5% [39].

NGS is also very suitable for detecting HLA loss. However, there are currently no commercial kits and analysis software available. For this purpose, the follow-up sample of the recipient is sequenced with NGS. Any SNP (or phased SNPs) in which the recipient-specific HLA allele differs from the donor-specific and shared allele can be used to calculate the proportion of cells carrying the recipient-specific allele.

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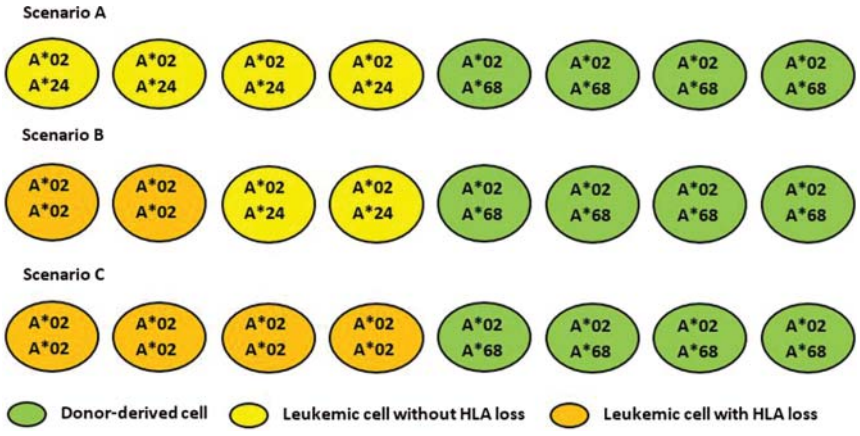

Figure 13.6 Different scenarios of inside HLA loss of leukemic cells after HLA mismatched stem cell transplantation for a patient with an outside-HLA chimerism of 50%.

The calculation is performed using the coverage of the SNPs or in case of phased SNPs with the read numbers. With this approach, the NGS can nearly always discriminate the recipient-specific allele and when using many different informative SNPs (or even better-phased SNPs). With NGS, an HLA loss can be detected in samples with less than 2% outside-HLA chimerism (own data).

Scenario A of Figure 13.6 shows a relapse without an HLA loss. In this scenario, the proportion of the recipient-specific allele (HLA-A*24) is 25%, with 50% of the cells carrying this allele.

Scenario B of Figure 13.6 shows a relapse with a partial HLA loss. In this scenario, the proportion of the recipient-specific allele (HLA-A*24) is 12.5%, with 25% of the cells carrying this allele.

Scenario C of Figure 13.6 shows a relapse with a complete HLA loss. In this scenario, none of the cells is carrying the recipient-specific HLA-A*24 allele.

An HLA loss is diagnosed when the inside-HLA chimerism (percentage of cells carrying recipient-specific HLA alleles) is significantly lower than the outside-HLA chimerism (% recipient chimerism). For most relapses with an HLA loss, the loss of the recipient-specific allele(s) is complete, which means that all leukemic cells have deleted the mismatched HLA locus.

13.12.2 Donor-Derived Cell-Free DNA in Solid Organ Transplantation

The detection of donor-derived cfDNA (dd-cfDNA) after SOT is a new noninvasive tool to identify graft damage. Subclinical or acute rejection, infections, ischemia/reperfusion, or toxic side effects of medication (e.g., calcineurin inhibitor toxicity) can lead to the release of cfDNA from the injured graft tissue [40]. Increased dd-cfDNA levels are helpful in diagnosing antibody-mediated rejection (AMR), but less supportive in diagnosing T-cell-mediated rejection.

During graft damage, the DNA is digested within the cells and is subsequently released as approximately 120–160 base pair pieces of double-stranded DNA into

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the blood [41]. cfDNA is quickly further degraded by DNAse enzymes present in the blood and cleared by the kidney and liver from the bloodstream, resulting in a half-life of just about 15–30 minutes [41–43]. When collecting, storing, and isolating cfDNA, some preanalytical measures have to be taken. The blood should be collected in special tubes, which preserve the cfDNA and stabilize the blood cells. In addition, the plasma should be collected by centrifugation within less than 24 hours in order to reduce "contamination" by DNA from the blood cells. Furthermore, the laboratory should have a DNA isolation method established, which can isolate low amounts of highly fragmented DNA. The principles for testing of dd-cfDNA do not differ that much from "normal" chimerism testing, including identifying informative markers and quantifying the dd-cfDNA in the plasma of the post-transplant sample. The most common method for testing dd-cfDNA seems to be NGS. dd-cfDNA can be reported as absolute concentration (copies per ml) or the ratio (percentage) of donor-derived to recipient-derived DNA. The latter is commonly used for clinical applications but has the disadvantage that other factors like surgery, injuries, necrosis, infarction, inflammation, and anaerobic exercise [44, 45] can impact on the ratio. Thresholds for identifying graft rejection differ from organ to organ and the size of the organ or number of the organs transplanted. The impact of the size can be seen when comparing single with double-lung transplantation, for which the optimal dd-cfDNA threshold for the detection of acute rejection can be nearly doubled (0.54% in single lung and 1.1% in double-lung transplantation) [46]. The median dd-cfDNA fraction in multiple kidney transplantation studies is significantly increased in patients with AMR (1.4–2.9%) compared to "stable" patients (0.21%), with a negative predictive value of 95% to rule out an ongoing rejection [47–49].

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14

Disease Associations with HLA Alleles

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14.1 HLA and Disease Association

14.1.1 Introduction to HLA and Disease Association

The first described association between a human leukocyte antigen HLA and a disease was Hodgkin's lymphoma with a group of HLA-B antigens (B5, B15, B18, B35) [1]. Shortly afterward, the first association of an autoimmune disease and a particular HLA type was reported. In 1973, two different groups published the association of ankylosing spondylitis with HLA-B27 [2, 3]. From that discovery until now, a large number of diseases have been observed to be associated to specific HLA antigens [4]. These associated diseases mainly correspond to autoimmune diseases, infectious diseases, cancer, or adverse reactions to drugs.

The main importance of defining an association between an HLA antigen and a disease is the possibility to characterize a population that can be at risk of developing a particular disease. Consequently, prevention procedures, if available, can be put into practice to this higher risk population. In addition, the knowledge that some genetic factors provide susceptibility to a disease can give valuable information on the pathophysiological mechanisms that drive its development and identify potential treatment targets.

14.1.2 Assessment of the Association Risk

The diseases associated to HLA antigens are caused by multifactorial events. Multiple genetic predisposing variants and environmental factors can influence the development of a disease. Therefore, even in diseases with a stronger association with an HLA antigen, there is no 100% correlation between the presence of the predisposing HLA antigen and the occurrence of the disease. For example, a major part of ankylosing spondylitis patients is B27, but the majority of B27-positive individuals are not going to experience ankylosing spondylitis during their lives.

Identification of disease genes can be achieved by means of the performance of family studies or by association population studies. However, the larger part of

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data linked to HLA is based solely on association studies that observe different frequencies of specific HLA alleles in disease individuals in relation to healthy control population. In this kind of study, the statistical analysis to determine if the frequency differences are significant is the chi-square test, using data from a 2×2 contingency table. If the frequencies are too small, the Fisher's exact test should be used instead.

Example of a generic 2×2 contingency table:

	Positive for HLA antigen	Negative for HLA antigen
Patients	a	b
Healthy controls	c	d

In order to measure the strength of the association, the risk of disease among positive individuals compared with the risk of those negative should be assessed. The estimation of the relative risk (RR) can be calculated using the following formula:

$$RR = \frac{ad}{bc}$$

An RR close to 1 means that there is no association between the antigen and the risk of disease. An RR greater than 1 implies that the individuals carrying the HLA allele are at an increased risk of having the disease (higher susceptibility to the disease), while an RR less than 1 implies that the positive individuals are at a lower risk of developing the disease (protection from the disease). A stronger association is present the further the number is higher or lower to 1.

It may seem easy to detect an association in a case–control population study, but it is not the case on many occasions. As the polymorphism in the HLA genes is extremely high and there is a strong linkage disequilibrium between the different HLA loci, it may be difficult to identify the actual allele or gene responsible for the association. In addition, in the human MHC region there are a lot of other genes, some with immune-related functions, which can be the actual genes associated to the disease. Furthermore, small sample case–control studies could obtain false-positive associations, which might not be confirmed by later studies.

Genome-wide association studies (GWAS) have sorted out many disease associations with MHC region variants [5]. GWAS have identified single-nucleotide polymorphism (SNP)-level associations for most robustly validated HLA associations, but detailed studies also show independent associations with non-HLA polymorphisms in the MHC region. However, there is still a degree of disappointment with the cumulative results since only a modest amount of disease heritability has been explained, even after multiple studies targeting the same disease.

14.2 Mechanisms of HLA and Disease Associations

14.2.1 Peptide Binding and Presentation

The primary mechanism underlying HLA disease associations is the differential binding and presentation of antigenic peptides. Specific HLA alleles bind particular

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peptides with varying affinities, influencing which antigens are presented to T cells. Each HLA allele has a unique peptide-binding groove that determines which peptides it can bind and present. This specificity is due to polymorphisms in the amino acid sequences of the HLA molecules, which shape the so-called binding pockets. Variations in these pockets influence the repertoire of peptides that each HLA allele can present to T cells.

This process can either enhance or diminish immune responses, contributing to disease susceptibility or protection. For example, in autoimmune diseases, certain HLA alleles may preferentially bind and present self-peptides that resemble foreign antigens, triggering an autoimmune response by autoreactive T cells, leading to tissue damage [6]. The efficiency of antigen presentation by HLA molecules influences the strength and quality of the immune response. HLA alleles that effectively present antigens can elicit robust T cell responses, which is beneficial for clearing infections but can also contribute to autoimmunity if self-antigens are presented. Conversely, alleles that present antigens poorly may lead to inadequate immune responses, increasing susceptibility to infections and possibly cancers.

In autoimmune diseases, autoreactive T cells escape the negative selection of the thymus and peripheral immune tolerance, attacking normal tissues. Autoreactive T cells may directly be generated and activated by several mechanisms altering the HLA-peptide-TCR binding [7], such as atypical HLA–peptide–TCR-binding orientation, low-affinity peptide binding that facilitates thymic escape, TCR-mediated stabilization of weak peptide–HLA interaction, and presentation of peptides in a different binding register.

Other mechanisms that may generate and activate autoreactive T cells are likely to be driven by epitope variation. These mechanisms are detailed in some of the following sections.

14.2.2 Molecular Mimicry

Molecular mimicry refers to the phenomenon where an HLA molecule presents a pathogen-derived peptide that closely resembles a self-peptide. This can result in cross-reactivity, where T cells activated by the pathogen peptide also recognize and attack the self-peptide. This mechanism is implicated in the development of autoimmune diseases. For instance, in rheumatoid arthritis, certain HLA-DRB1 alleles contain a shared epitope motif that binds peptides derived from microbial antigens as well as self-peptides from joint tissues. The immune response against these microbial peptides can inadvertently target the joints, leading to chronic inflammation and joint damage.

Molecular mimicry is not limited to direct peptide similarities but can also involve broader immune activation. Infections can induce bystander activation of autoreactive T cells or alter the immune environment in ways that promote autoimmunity. For example, Epstein–Barr virus infection is associated with multiple sclerosis, and the presence of certain HLA alleles (e.g., HLA-DRB1*15:01) may facilitate the presentation of Epstein–Barr virus antigens that resemble myelin proteins, contributing to the autoimmune attack on the central nervous system.

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14.2.3 Epitope Spreading

Epitope spreadingoccurs when an immune response initially directed against a specific epitope expands to target additional epitopes. This process can aggravate autoimmune diseases by broadening the range of self-antigens recognized by the immune system. This can take place when an initial immune response to a particular self-antigen expands to include other self-antigens. HLA alleles that effectively present multiple self-peptides may facilitate this process, exacerbating disease progression.

In the case of systemic lupus erythematosus, the initial immune response may target nuclear antigens released from apoptotic cells. Over time, the immune response can spread to other nuclear components, increasing the breadth of autoantibodies and the severity of the disease. HLA alleles that are able to present multiple related self-peptides can amplify the autoimmune response.

14.2.4 Epigenetic and Post-Translational Modifications

Environmental factors can also influence gene expression through epigenetic modifications, which can affect the presentation of antigens by HLA molecules. For example, infections, diet, and stress can alter the expression of HLA genes or the processing of antigens, modulating the immune response and influencing disease outcomes. Aberrant methylation patterns can lead to the inappropriate expression of autoantigens or immune-regulatory genes, creating a permissive environment for autoimmunity. Furthermore, changes in histone modifications can alter chromatin structure and gene expression, potentially leading to the expression of cryptic antigens that are normally hidden from the immune system. For instance, in systemic lupus erythematosus aberrant DNA methylation and histone modifications can result in the expression of autoantigens and subsequent immune complex formation. Another potential example of an epigenetic effect involving HLA is the parent-of-origin effect reported in multiple sclerosis. It has been observed that offspring of affected mothers are more likely to develop the disease than those of affected fathers. The effect has been localized to *HLA-DRB1*15*, the major risk allele for multiple sclerosis.

On the other hand, post-translational modifications such as phosphorylation, methylation, citrullination, or acetylation have been shown to produce self-generated neoantigens from existing proteins and peptides by altering the primary structure of proteins, without the need to introduce antigens from microbial and somatic mutations. Post-translational modifications may allow the binding of a peptide to an HLA molecule that otherwise would not be able to optimally fit in its binding pocket. For example, in rheumatoid arthritis citrullinated proteins are presented by HLA-DRB1*04:01 or other HLA alleles, leading to T cell activation and joint inflammation. Or, in multiple sclerosis, post-translationally modified myelin proteins can be presented by HLA-DRB1*15:01, triggering autoreactive T cell responses against myelin.

The generation of neo-epitopes through epigenetic and post-translational modifications can lead to their presentation by HLA molecules, which can then be recognized by autoreactive T cells. Therefore, these modifications can create novel

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antigenic determinants, break immune tolerance, and lead to the activation of autoreactive T cells, driving the autoimmune process.

14.2.5 Linkage Disequilibrium with Non-HLA Genes

Many genes are present within the MHC region in addition to those that encode the HLA antigens. Some of these genes encode proteins with significant relevance to the immune system, such as complement components (C2, C4, and factor B), proteasome units, TNF, or the heat shock protein hsp70. Therefore, it is possible that polymorphisms of these non-HLA genes are the true susceptibility factors related to the association with the disease. The apparent HLA association may only reflect the high linkage disequilibrium between allelic variants of the non-HLA and the HLA genes.

In addition, some nonimmune-related genes are also located in the MHC region, and these could be responsible for the association found between HLA and some diseases. For example, it has been known for many years that hereditary hemochromatosis, a rare disorder of iron metabolism, was linked to the HLA-A3 antigen. Nevertheless, a gene called HFE is most often the cause of hereditary hemochromatosis. Having two copies of an altered HFE gene, which has two common mutations (C282Y and H63D), is the greatest risk factor for this disease. In this way, most Caucasian patients with hereditary hemochromatosis are homozygous for HLA-A3 and for the C282Y mutation of the HFE gene. In fact, the gene HFE is located on chromosome 6 near the HLA-A locus, and this specific mutation of the HFE is in strong linkage disequilibrium with the HLA-A3 variant.

14.3 Autoimmune Diseases Associated with HLA

Some of the most relevant autoimmune diseases associated to HLA antigens are listed in the following table:

Disease	HLA antigen	Relative risk
Ankylosing spondylitis	HLA-B27	~87
Reactive arthritis, including Reiter syndrome	HLA-B27	~37
Behçet's disease	HLA-B51	~4
Birdshot chorioretinopathy	HLA-A29	~109
Psoriasis	HLA-Cw6	~8–10
Celiac disease	HLA-DQ2 (DQA1*05/DQB1*02)	~35
	HLA-DQ8 (DQA1*03/DQB1*03:02)	~5–10
Type 1 diabetes	HLA-DR3/DQ2	~3–5
	HLA-DR4/DQ8	~4–8
	HLA-DR3/DR4	~20
	HLA-DR15/DQ6	~0.15–0.19
Multiple sclerosis	HLA-DR2 (DRB1*15)	~5
Systemic lupus erythematosus	HLA-DR3	~2–6
Rheumatoid arthritis	HLA-DR4	~4–10
Narcolepsy	HLA-DRB1*15/DQB1*06:02	~130

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Generally, HLA class I-associated autoimmune diseases are seronegative, while HLA class II-associated autoimmune diseases are accompanied by the presence of autoantibodies. In fact, the importance of HLA class II polymorphisms in the emergence of autoantibodies has been observed in many studies, with distinct T cell specificities accounting for the association of different HLA alleles with particular autoantibodies (for instance, in celiac disease HLA-DQ2 or DQ8 is required for the generation of anti-transglutaminase 2 antibody) [8]. In addition, there is heterogeneity on the diseases with different immune responses contributing to the pathogenesis, such as in rheumatoid arthritis, where anti-citrullinated protein antibody positive and negative diseases are genetically distinct.

14.3.1 Ankylosing Spondylitis and HLA-B27

Ankylosing spondylitis is a chronic, inflammatory disease that mainly affects the axial skeleton and sacroiliac joints. It is a male-predominant disease, unlike most autoimmune diseases, and its genetic heritability is extremely high, with estimates exceeding 90%. HLA-B27 has the strongest association with ankylosing spondylitis, especially in Caucasians, with 88–96% of patients being positive [2, 3]. Ethnic differences seem to exist regarding disease susceptibility conferred by HLA-B27. The RR of developing ankylosing spondylitis in HLA-B27 positive individuals is increased 20- to 100-fold in the Caucasian population. In addition, 30–80% of patients with reactive arthritis and 20–35% of patients with psoriatic arthritis are also HLA-B27 positive.

Most of the relatively common HLA-B27 alleles, such as B*27:02, B*27:04, B*27:05, and B*27:07, have been shown to be associated with ankylosing spondylitis, although some alleles, namely B*27:03, B*27:06 (both common in Southeast Asian populations), and B*27:09 (found in Sardinia and southern Italy), may be exceptions. Changes in the primary structure of the HLA-B27 protein could explain the different levels of association. Particularly, the variations on the antigen-binding cleft can consequently affect the peptide specificity. However, the specific mechanism by which HLA-B27 predisposes to this disease remains elusive despite intensive research in the last decades. Neither one of the existing hypotheses can fully describe and explain the underlying mechanisms [9]. The "arthritogenic peptide" hypothesis proposes that the structure of the molecule encoded by disease-associated HLA-B27 alleles enables presentation of microbial peptides that display molecular mimicry with specific arthritogenic self-peptides. Then, HLA-B27-restricted cross-reactive cytotoxic T-cell could respond against self-peptides, resulting in chronic inflammation, but definitive role of microbial antigens or arthritogenic peptides in the pathogenesis of ankylosing spondylitis has not been unequivocally demonstrated.

However, other possible mechanisms have been described. HLA-B27 heavy chain tends to misfold in the endoplasmic reticulum due to the ability of HLA-B27 heavy chains to form stable homodimers. Misfolded heavy chains can accumulate in the endoplasmic reticulum, leading to a stress response, cytokine production by macrophages and inflammation. β2-microglobulin-free HLA-B27 heavy chain homodimers can also be found on the cell membrane, where they can interact with

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CD4-positive T lymphocytes, NK cells, and monocytic cells that express KIR and LILR receptors. Additionally, β2-microglobulin can also be released from HLAB27 molecules on the cell surface and be deposited in synovial tissue. Therefore, while the arthritogenic peptide hypothesis presents a model compatible with classic autoimmunity, the unfolded protein response hypothesis as well as the cell surface HLA-B27 homodimers hypothesis support the autoinflammatory aspects of the disease.

HLA-B27 typing is performed in many laboratories for help in the diagnosis of ankylosing spondylitis as more than 96% of ankylosing spondylitis patients are HLA-B27 positive, as opposed to a 5–7% prevalence of HLA-B27 in Caucasoid populations. Nevertheless, screening of the general population is not recommended as a positive result only indicates genetic susceptibility and only a minority of HLA-B27 carriers will develop the disease. Therefore, even though positivity for HLA-B27 can be a useful confirmatory marker, it is not diagnostic. More importantly, its absence virtually excludes a diagnosis of ankylosing spondylitis. In addition, HLA-B27 typing may also play a useful role in the diagnosis of other HLA-B27-associated diseases, such as Reiter's disease.

14.3.2 HLA-B51 and Behçet's Disease

Behçet's disease is a chronic systemic vasculitis characterized by the presence of recurrent oral and genital ulcers, skin lesions, and uveitis. Its exact etiology is unknown, but it is believed to be the result of a combination of genetic and environmental factors. Various studies have shown that the HLA-B51 antigen is significantly associated with an increased risk of developing Behçet's disease [10]. This association has been particularly evident in populations from regions where the disease is more prevalent, such as in countries in the Mediterranean basin, the Middle East, and East Asia. In some studies, HLA-B51 can increase the risk up to six times compared to individuals who do not carry this allele.

Although the exact mechanism in which HLA-B51 contributes to Behçet's pathogenesis is not completely clear, it is postulated that it could involve the presentation of autoantigens or microbial peptides that trigger an aberrant autoimmune response. It is also suggested that HLA-B51 could influence NK cell activity and the production of proinflammatory cytokines.

The presence of HLA-B51 can support the diagnosis of Behçet's disease, especially in cases where the symptoms are atypical or not all the classic diagnostic criteria are met. Some studies suggest that HLA-B51-positive patients may have a more severe course of the disease, although this is not universally accepted. The screening of high-risk populations is not recommended as most HLA-B51 carriers do not develop Behçet's disease. Moreover, Behçet's disease cannot be excluded in the absence of HLA-B51.

14.3.3 Celiac Disease and DQ2/DQ8

Celiac disease is an autoimmune condition of thesmall intestine that is triggered by the ingestion of gluten in genetically predisposed people. Successful treatment

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is avoidance of dietary gluten. Long-standing evidence suggests a T cell-mediated response to peptides derived from the gliadin fraction of wheat gluten, leading to immunologically mediated intestinal injury in genetically susceptible individuals. The relationship between celiac disease and the HLA class II DQ2 and DQ8 alleles is well known and crucial for the diagnosis and understanding of the pathogenesis of the disease. A link with non-MHC genes has also been described in GWAS.

DQ2.5 and DQ8 antigens, encoded by the disease-associated DQA1/DQB1 heterodimers (DQA1*05:01,DQB1*02:01 and DQA1*03,DQB1*03:02), present modified peptides coming from native gliadin-derived peptides [11]. The tissue transglutaminase enzyme, released during tissue damage in celiac disease, can convert noncharged glutamine residues to negatively charged glutamic acid. This deamidation process generates modified peptides with negative changes at proper positions for binding to DQ2.5 and DQ8. As a result, the gluten-specific $CD4^+$ T-cell repertoire is substantially expanded, enhancing inflammation and development of disease. Inflammation causes the recruitment of other immune cells, including intraepithelial lymphocytes, which also contribute to mucosal damage. The activation of both the innate and adaptive immune system leads to TNF-alpha and -Interferon-gamma secretion, which ultimately result in tissue damage, including the destruction of intestinal villi that are essential for the absorption of nutrients, and disease manifestations. Activation of B cells also leads to the production of specific antibodies against tissue transglutaminase and gliadin peptides. In fact, these antibodies are useful for the diagnosis of celiac disease. The composition of the gut microbiota may also have some influence in the predisposition and severity of the disease.

According to the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition guidelines, for the diagnosis of celiac disease testing of HLA-DQ2/DQ8 should be included in the diagnostic workup of celiac disease in children [12]. Biopsy of the small bowel may not be necessary in the pediatric population in case of symptomatic patients with high transglutaminase antibody levels and HLADQ2/DQ8 positivity. HLA testing may also be useful to exclude disease in high-risk individuals, such as first-degree relatives of celiac disease patients or patients with related diseases. As testing of DQ2/DQ8 has a high negative predictive value of 99%, a negative test virtually excludes celiac disease diagnosis, while a positive test just indicates genetic susceptibility. Ninety percent of patients with celiac disease express HLA-DQ2.5 *in cis*, encoded by HLA-DQA1*05:01-DQB1*02:01, or DQ2.5 *in trans*, arising from the HLA-DQA1*05:05-DQB1*03:01/DQA1*02:01-DQB1*02:02 haplotypes. Five percent of the patients carry HLA-DQ8, from the HLA-DQA1*03:01-DQB1*03:02 haplotype. Patients negative for these HLA molecules frequently hold the HLA-DQA1*02:01-DQB1*02:02 haplotype (HLA-DQ2.2).

14.3.4 Type 1 Diabetes Mellitus

Type 1 diabetes mellitus (T1DM), also known as insulin-dependent diabetes, is an autoimmune disease characterized by the destruction of pancreatic beta cells,

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resulting in insulin deficiency. There is a strong genetic association between T1DM and certain alleles of the HLA system. HLA-DR3 and HLA-DR4 antigens are strongly associated with an increased risk of developing T1DM. In fact, in European Caucasoid populations, over 95% of T1DM patients are of DR3 or DR4 genotype, and of these 60% are heterozygotes for DR3/DR4. However, extensive studies have indicated that HLA-DQ alleles are largely responsible for this T1DM susceptibility [13, 14]. Thus, HLA-DQ2 (DQA1*05:01-DQB1*02:01) and HLA-DQ8 (DQA1*03:01-DQB1*03:02) antigens are highly associated with an elevated risk of T1DM. Heterozygosity for both risk haplotypes DQ2/DQ8 confers the greatest known genetic risk for T1D. On the other hand, DQB1*06:02 is a strong protective allele. Although most research has focused on class II alleles, class I alleles such as HLA-A and HLA-B also play a role in susceptibility to T1DM. For example, HLA-B8 and HLA-B18 have been also associated with the disease. While GWAS reliably show that the strongest linkage happens between the HLA complex and T1DM, more than 40 additional non-HLA polymorphisms have been shown to be associated with T1DM, all of them with small effects on disease susceptibility. Although HLA alleles confer a strong genetic predisposition, the development of T1DM is also influenced by environmental factors such as viral infections, diet, and other immunological factors. The interaction between genetic predisposition and environmental factors determines the real risk of developing the disease.

Predisposing and protecting DQ antigens can be differentiated by the amino acid present at position 57 of the DQβ chain. Position 57 is believed to be a critical residue in peptide binding pocket P9 and the presence or absence of aspartic acid at this position could therefore affect the stability of the DQ molecule and the repertoire of peptides, which can be bound and presented to T cells. DQ molecules with no aspartic acid at this position 57 have greater potential for binding and presenting peptides from certain autoantigens, leading to an autoreactive T-cell response. Despite these well-described HLA-DQ associations with T1DM susceptibility or resistance and a nice molecular model to support the association, identification of potential β cell autoantigen-derived peptides or modified peptides has been problematic. However, it has been possible to show that a single insulin epitope (InsB13-21) can bind to DQ molecules encoded by the DQ2/DQ8 heterozygous genotype, resulting in a proinflammatory T-cell response. On the other hand, another insulin peptide (InsB6-14) can bind to the DQ molecule encoded by the protective DQ6 antigen, resulting in an anti-inflammatory response.

One additional factor that might contribute to the associations between DR–DQ haplotypes and T1D is the intrinsically protein instability of DQ. It has been reported that DQA1*05-DQB1*02 and DQA1*03-DQB1*03:02 haplotypes generate SDS unstable DQ proteins, whereas the protective haplotype DQA1*01:02-DQB1*06:02 generates an SDS stable DQ protein. An intrinsically unstable HLA molecule may increase the risk for T1D through facilitating the formation of unstable HLA-self peptide complex, which may permit the thymic escape of self-reactive T cells [15]. The associations of T1D susceptible/protective DR–DQ haplotypes with a variety of other autoimmune diseases indicate that the mechanism of autoimmune susceptibility and/or protection may partly be shared among certain autoimmune diseases. As

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distinct sets of self-peptides are involved in individual diseases, it would not be possible to explain the shared associations by the peptide-binding spectrum of DR and DQ proteins. The HLA stability model or other molecular mechanisms, which could operate irrespective of the presented self-peptides, may underlie these associations.

In spite of the strong genetic associations between specific HLA-DR and DQ antigens and T1DM, the use of HLA testing for disease prediction is not performed routinely. When a family member is diabetic, the antibody testing is very accurate for predicting the likelihood of disease and, therefore, the predictive value from using HLA typing as a screening tool is limited. Additionally, screening in the general population lacks specificity as the associated DR-DQ alleles are common in the unaffected population.

14.3.5 Rheumatoid Arthritis

Rheumatoid arthritis is a chronic autoimmune disease that causes inflammation, pain, and joint destruction. There is a strong genetic association between rheumatoid arthritis and certain HLA class II alleles, especially at the HLA-DRB1 locus. HLA-DRB1*04 alleles (particularly HLA-DRB1*04:01, DRB1*04:04, and DRB1*04:05) are strongly associated with an increased risk of developing this disease as these alleles are present in a significant percentage of patients with rheumatoid arthritis. Other associated alleles that also confer susceptibility to the disease are HLA-DRB1*01, DRB1*10, and DRB1*14:02. The HLA-DRB1 alleles associated with rheumatoid arthritis share a specific epitope motif in the 70–74 region of the beta chain of the HLA-DR molecule, known as the "shared epitope" [16]. This motif appears to be crucial for the presentation of certain antigenic peptides to T lymphocytes, triggering its characteristic autoimmune response. Although most associations are found in class II alleles, some studies have also identified associations with HLA class I alleles, such as HLA-B and HLA-C, although these associations are less strong and less well understood.

The relationship between HLA and rheumatoid arthritis is based on the role of HLA molecules in presenting antigens to T lymphocytes, which is crucial for the adaptive immune response. HLA-DRB1 molecules with the shared epitope present peptides derived from joint proteins (such as type II collagen) or citrullinated peptides (peptides that have undergone post-translational modification) to $CD4^+$ T cells. This presentation can activate autoreactive T lymphocytes. Citrullination is a process in which arginine in proteins is converted to citrulline. In rheumatoid arthritis, T cells can recognize citrullinated peptides presented by HLA-DRB1 with the shared epitope, leading to an immune response against citrullinated proteins in the joints. The activation of $CD4^+$ T cells can lead to the activation of B cells and the production of autoantibodies such as rheumatoid factor and antibodies against citrullinated peptides. These autoantibodies contribute to inflammation and joint damage. Infections, smoking, and other environmental factors can interact with genetic predisposition to trigger or exacerbate rheumatoid arthritis. For example, smoking has been associated with an increased risk of disease in individuals with HLA-DRB1 alleles with the shared epitope.

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HLA typing does not play a significant role in the diagnosis of rheumatoid arthritis. Approximately 70% of Caucasoid rheumatoid arthritis patients carry at least one DRB1*04 shared epitope allele compared with a population prevalence of approximately 35%. As a result, the RR of the disease is too low to be of diagnostic value. Conversely, genotyping for shared epitope alleles may be helpful in identifying poor prognosis patients with severe erosive forms of synovitis, thus these patients may benefit from early therapeutic intervention.

14.3.6 Narcolepsy Type 1

Narcolepsy type 1 is a rare, chronic, and disabling neurological disease causing excessive daytime sleepiness and cataplexy. This disease was initially described in Japan to be tightly associated with the HLA-DR2 haplotype (DRB1*15:01-DQB1*06:02) [17]. Similar findings were also made in other populations, but other HLA haplotypes containing the DQB1*06:02 allele were also associated, indicating that HLA-DQB1 rather than HLA-DRB1 plays a primary role in susceptibility to this disease. In fact, 98% of patients worldwide with undetectable levels of hypocretin in cerebrospinal fluid are DQB1*06:02 positive, which makes narcolepsy type 1 one of the diseases with the strongest HLA association, having an odds ratio of approximately 200 in some ethnic groups.

While the exact etiology of narcolepsy type 1 is still unknown, many studies have provided convincing evidence supporting its autoimmune origin. The prevailing hypothesis on its pathogenesis involves an immune-mediated loss of neurons producing the hypocretin (also known as orexin) neuropeptides in the lateral hypothalamus that can be triggered by vaccination and/or by infection in genetically susceptible patients [18]. The search for both self-antigens and foreign antigens recognized by the pathogenic T cell response in this disease is still ongoing. Increased T cell reactivity against these neurons has been consistently reported in patients with narcolepsy type 1, but data demonstrating a primary role for T cells in neuronal destruction are still currently lacking.

HLA analysis can be part of the diagnosis of narcolepsy, especially when narcolepsy type 1 is suspected. However, having the HLA-DQB1*06:02 allele is not definitive for the diagnosis, as it is also frequently present in healthy individuals. Definitive diagnosis is usually based on a combination of clinical history, sleep tests, such as polysomnography and multiple sleep latency testing, and measurement of hypocretin levels in the cerebrospinal fluid.

14.4 Other Diseases Associated with HLA

Aside from autoimmune diseases, other diseases are also associated with specific HLA antigens. Generally speaking, all of these diseases have in common the fact that presentation of the antigen by HLA molecules and the subsequent activation of the immune response are a central feature of their pathophysiology.

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These other types of diseases that are described to be associated with HLA antigens are infectious diseases, cancer, or drug hypersensitivity. A chapter of this book is dedicated to this later group of diseases in relation to the susceptibility to adverse reactions to drugs provided by certain HLA alleles. Conversely, a brief review of the associations with infectious diseases and with cancer is included below.

14.4.1 Infectious Diseases

The association between HLA and infectious diseases is generally explained by the ability of different HLA alleles to present viral or bacterial peptides to T cells with different effectivity. An effective presentation can result in a robust immune response and more rapid clearance of the pathogen, while an ineffective presentation can lead to persistent infection or more severe disease. The relationship between HLA and infectious diseases is complex and can vary significantly between different populations and epidemiological contexts. Furthermore, the interaction between genetic and environmental factors also plays a crucial role in determining susceptibility and resistance to infections. In addition, viral or bacterial antigens can evolve over time and between strains, so the antigens do not have an immutable amino acidic sequence to be presented by the HLA molecules. Overall, the association of the different infectious diseases with HLA alleles is not as strong as in the case of autoimmune diseases, presenting quite low RRs.

The huge genetic variation observed in HLA genes in human populations is principally considered the result of an adaptation to pathogen-rich environments during evolution. Heterozygosity at the HLA genes confers an advantage to individuals, which would be capable of presenting a larger variety of peptides than in homozygous individuals, therefore enhancing their T cell repertoire and protection against pathogens. On the other hand, protective or harmful effects of specific HLA alleles have been suggested through several epidemiological approaches related to infectious diseases [19]. In fact, there is a need to better understand how everyone reacts against different kinds of infections considering their HLA genotype. From an evolutionary point of view, it is also very relevant to understand how specific HLA genes evolved in relation to the spread of infectious diseases in different populations. For example, in the case of a severe epidemic leading to a reduction in size of a given population, a population bottleneck, it is expected that highly protective alleles are maintained in the population through positive selection while susceptibility alleles are eliminated through purifying selection or selective sweep.

Here are some examples of the main known infectious diseases associated to HLA antigens:

- **HIV/AIDS:** Some HLA antigens, such as HLA-B*27 and HLA-B*57, have been associated with slower disease progression in people infected with HIV. These alleles are capable of presenting HIV epitopes to cytotoxic T cells (CTLs) more efficiently. This allows for a robust immune response against the virus, reducing its replication. The presence of HLA-B*27 or HLA-B*57 is correlated with a lower viral load, a better preservation of $CD4^+$ T cells, and, consequently, a slower progression to AIDS in people infected with HIV. On the other hand, antigens such as

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HLA-B*35 have been linked to a more rapid progression to AIDS and an increased mortality associated with HIV infection. This allele seems to be less effective in presenting critical viral epitopes to CTLs. This may allow for faster virus replication and greater destruction of $CD4^+$ T cells.

It should be highlighted that the selective pressure exerted by HLA alleles can lead to the evolution of HIV viral variants that evade the immune response. HLA alleles that allow a greater diversity of epitopes presented may limit the ability of the virus to escape. Furthermore, the identification of HIV epitopes presented by HLA alleles associated with better viral control may guide the design of vaccines that induce similar immune responses.

- **Hepatitis B:** HLA antigens such as HLA-DQ2 and HLA-DQ8 have been associated with an increased susceptibility to chronic hepatitis B virus infection. On the contrary, HLA-DRB1*13 has shown an association with a higher rate of virus clearance, suggesting resistance to chronic infection.
- **Hepatitis C:** The HLA-DRB1*11 allele has been associated with a higher likelihood of spontaneous clearance of hepatitis C virus. Other HLA class I antigens such as HLA-B*27 and HLA-B*57 have also been associated with a better response to antiviral therapy.
- **COVID-19:** The association between COVID-19 and HLA types has been a subject of research to understand the individual differences on the response to SARS-CoV-2 infection. Certain HLA types have been linked to either increased susceptibility to SARS-CoV-2 infection or a higher likelihood of severe COVID-19 outcomes. While HLA allele frequencies vary by population, potentially explaining regional differences in COVID-19 impacts, various studies have identified alleles, such as HLA-DRB1*01:01 and HLA-A*11:01, that seem to be associated with COVID-19 severity. It is important to note that the relationship between HLA and COVID-19 is complex, influenced by other genetic, environmental, and immunological factors. In addition, results can vary based on study design, population sampled, and analysis methods.
- **Malaria:** Some HLA alleles, such as HLA-B*53, have been shown to be associated with increased resistance to severe malaria caused by *Plasmodium falciparum*. Other studies suggest that diversity in HLA alleles may confer an evolutionary advantage in malaria-endemic areas.
- **Tuberculosis:** Alleles such as HLA-DRB1*04 and HLA-DRB1*10 have been associated with increased susceptibility to tuberculosis. In contrast, HLA-DRB1*15 has been shown to be associated with lower susceptibility in certain population groups.

14.4.2 Cancer

Genetic variability in HLA genes significantly influences individual susceptibility to various cancers. The diversity of HLA alleles within the population means that different individuals may present and recognize tumor antigens differently, affecting their immune response to cancer cells [20].

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Some examples and mechanisms through which HLA genetic variability can impact cancer susceptibility may be

- **Cervical cancer:** HLA-DRB1*13 has been associated with a reduced risk of cervical cancer. Studies suggest that this allele may enhance the presentation of human papillomavirus (HPV) antigens, leading to a more effective immune response against HPV-infected cells, which are the primary cause of cervical cancer.
- **Nasopharyngeal carcinoma:** An increased risk of nasopharyngeal carcinoma has been linked to the HLA-B27 allele. This cancer is commonly associated with Epstein–Barr virus (EBV) infection, and HLA-B27 might influence the presentation of EBV antigens, altering immune surveillance capabilities.
- **Melanoma:** Various HLA class I alleles, such as HLA-A*02 and HLA-A*24, have been observed to be associated with melanoma risk. Some alleles may be more efficient at presenting melanoma-associated antigens, affecting the effectiveness of the immune response.
- **Leukemia and lymphoma:** Specific HLA alleles and haplotypes have been implicated in the risk of developing hematological malignancies. For example, HLA-DRB1*15 has been associated with an increased risk of Hodgkin's lymphoma. The mechanism may involve differential antigen presentation that impacts immune recognition and response to malignant cells.
- **Breast cancer:** Certain alleles, such as HLA-DQB1*03, have been associated with an increased risk of breast cancer. Research suggests that variations in HLA class II genes may affect the immune system's ability to recognize and destroy early-stage tumor cells in breast tissue.
- Other cancers (colorectal cancer, gastric cancer, prostate cancer) have also been linked with different HLA class I or II alleles through similar mechanisms.

In addition, the presence of certain HLA polymorphisms can influence the prognosis of cancer patients by affecting the effectiveness of the immune response against the tumor. For example, the presence of specific HLA alleles might correlate with better responses to treatments like checkpoint inhibitors. In fact, HLA typing is essential for the success of immunotherapies, such as adoptive T cell therapies and cancer vaccines, as the effectiveness of these treatments often depends on the compatibility of HLA molecules between donors and recipients.

14.5 Laboratory Diagnostics

14.5.1 Clinical Utility

Even though a high number of diseases have been described to be associated to HLA antigens, in most of these diseases it makes no sense to use this information at the clinical level. In some cases, the low RR of the association precludes its utility since the small susceptibility increase that confers the presence of the associated HLA allele does not proportionate any help for the diagnosis of the disease. In other cases, the diagnosis can be obtained by other means, clinical or analytical, so the knowledge about the presence of the associated HLA allele is not useful either.

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The main value of the assessment of the associated HLA antigens rests on those diseases associated to an HLA antigen with a high RR. Despite that, even in these strongly associated diseases, the presence of the HLA-associated allele in a patient is not diagnostic of the disease. It is important to bear in mind that the majority of individuals who carry the HLA-associated allele will never develop the disease since the specific HLA allele can be quite common in the population. On the other hand, the absence of the HLA-associated allele in a patient virtually excludes the diagnostic of the disease. It is still possible that some affected patients do not carry the associated allele, but as the majority of patients will have it, a negative detection indicates a low probability of developing the disease.

In diseases associated with a low RR, the determination of the HLA allele could still have some value when prognosis can be also associated, for instance, in rheumatoid arthritis, or in the study of family members of an affected patient, such as in T1DM.

14.5.2 Typing Techniques Used for the Assessment of Associated HLA Antigens

Techniques currently in use to obtain the typing of HLA loci are explained in other chapters of this book. Nevertheless, in clinical practice, the purpose in the field of HLA and disease association is not to get a complete HLA typing of a patient but to know if the patient has or does not have the specific HLA allele that confers susceptibility to a disease. Therefore, techniques meant to simply assess the presence of a particular HLA antigen have been extensively developed. For example, in order to help on the diagnosis of ankylosing spondylitis, techniques assessing the presence or absence of HLA-B27 are mostly used because of their simplicity to perform and to interpret. In this sense, DNA-based techniques with PCR reactions specific for the amplification of a particular antigen, such as PCR-SSP (sequence-specific primers) or real-time PCR, are commonly applied.

For research purposes, the main HLA typing technique currently is nucleotide sequencing by next-generation sequencing (NGS), as this method is able to provide high-resolution results in all HLA loci. The comprehensive HLA typing results obtained by NGS make possible the discovery of associations not previously described or improve in the knowledge of the specificity of already known associations. Imputation of classical HLA type to four-digit resolution based on high-density SNP genotyping is an accurate, fast, and cost-effective alternative that is suitable for analyzing large cohorts and allows the integration with genome-wide association analysis. This is especially relevant for polygenic diseases involving more than one HLA gene and with interactions with other non-HLA genes.

14.6 Concluding Remarks

Major progress has been made in the description of the molecular mechanisms through which HLA proteins confer susceptibility to autoimmune diseases. Nevertheless, despite improvement in identifying the specific HLA genes and

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alleles implicated in disease risk and resistance through large case–control cohort analyses, a more high-throughput determination of the molecular and cellular basis of these associations with respect to the exact antigens and TCRs involved is still a major aim for the future. A careful analysis of HLA association with autoimmune disease may be of great interest for the understanding of the disease pathophysiology, the development of new treatments that could benefit patients with these diseases, and the identification of at-risk individuals who may be included in such prophylactic or therapeutic strategies.

The results of individual HLA and disease associations analysis in patients should be interpreted with caution since a positive result merely indicates genetic predisposition and is not suitable for the diagnosis of the associated disease. On the other hand, while the absence of a strongly associated HLA allele in a patient virtually excludes the diagnostic of the disease, this cannot absolutely be excluded, especially in non-Caucasian patients, as all associated diseases are multifactorial, with other genetic and environmental factors importantly contributing on their development.

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15

Drug Associations with HLA Alleles

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Interindividual differences in drug response are a common incident in pharmacological therapy. While some patients respond as anticipated, in others (an estimated 10–45% of patients), it can result in a lack of efficacy or adverse reactions [1]. An estimated 20–30% of the observed variability in drug response is explained by genetic germline variations in genes that are involved in pharmacokinetics and pharmacodynamics [2]. Many of these pharmacogenes are among the most polymorphic genes in the human genome with thousands of genetic variants, which can change enzyme activity or disrupt drug–target interactions, thereby eventually altering drug effects. This chapter offers an overview of the clinically significant links between specific human leukocyte antigen (HLA) alleles and drug-induced hypersensitivity reactions (HSRs).

15.1 Introduction

Over the last two decades, more and more research projects investigated the association of HLA alleles with drug HSRs, and specific HLA class I alleles have been strongly associated with T-cell-mediated drug HSRs. The growing knowledge of the immunological, pharmacological, and genetic mechanisms underpinning HLA-associated drug reactions can reduce the risk of severe reactions and set the course for personalized medicine approaches.

Pharmacogenomics, the study of how genes affect a person's response to drugs, has provided critical insights into drug safety and efficacy. While pharmacogenomics investigates how multiple variants across the genome affect drug response or reflect the combination of genetic variants and gene expression profiles, immunopharmacogenomics refers to effects involving inherited variants in genes important for the immune system. Among the genetic markers of interest, the HLA system stands out due to its strong association with drug HSRs or adverse drug reactions (ADRs).

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ADRs are the major causes of hospital admissions and readmissions, thus representing the leading causes of morbidity, mortality, and excess costs. In Europe, it is estimated that 5% of all hospital admissions and 197,000 fatal cases annually are associated with ADRs. The economic burden of ADRs on society is approximately $177 billion in the USA and €79 billion in Europe [3–6].

ADRs have traditionally been classified into two main categories: type A reactions, which are predictable and related to the drug's pharmacology, and type B reactions, which are idiosyncratic, often triggered by drug-induced immune activation, and linked to an individual's genetic variations [7–10].

Both type A and B reactions have several subcategories [7, 11]. While signs, symptoms, and the timing of onset can aid in distinguishing between these types of reactions, there may be some clinical overlap.

Type A reactions are common and account for 85–90% of ADRs. These reactions include drug interactions, overdoses, and predictable or dose-dependent side effects, such as diarrhea after using antibiotics or gastritis following the use of nonsteroidal anti-inflammatory drugs (NSAIDs) [7, 12].

The idiosyncratic type B ADRs represent the minority (up to 13%) of ADRs and are typically more severe than the predictable type A ADRs [14]. This group includes HSRS, drug-induced autoimmunity, pseudoallergic reactions (vancomycin flushing syndrome), and idiosyncratic reactions that are not mediated by immunological or inflammatory mechanisms (e.g. dapsone-induced hemolysis in G6PD-deficient patients) [7, 12]. As most type B reactions are immune-mediated, they are classified as hypersensitivity drug reactions (HDRs). HSRs can be further categorized as either immediate or delayed, depending on the timing of symptom onset. [6, 15] "Immediate reactions" occur shortly after drug administration (<1 hour), are IgE-mediated, and clinical symptoms include angioedema, urticaria, bronchospasm, and anaphylaxis. "Delayed HSRs" typically appear days or even weeks after drug exposure and are mediated by T lymphocytes. Delayed-type HDRs include a heterogeneous spectrum of systemic syndromes and organ-specific manifestations, with the most notable being cutaneous reactions and liver injury [6, 15, 16].

Since some ADRs do not fit into the traditional classification, such as chronic adverse effects related to cumulative drug exposure (e.g. osteoporosis with long-term corticosteroid use) or withdrawal reactions (e.g. rebound hypertension after discontinuing centrally acting antihypertensive drugs), an alternative and potentially more comprehensive classification system, known as "DoTS," has been proposed. DoTS classifies reactions dependent on the **Do**se of the drug, the **T**ime course of the reaction, and relevant **S**usceptibility factors (genetic, pathological, and other biological differences) [17]. In addition to classifying reactions, the DoTS system is valuable for considering the diagnosis and prevention of ADRs in clinical practice.

15.2 Classification of Drug Hypersensitivity Syndromes

The drug hypersensitivity syndromes (DHS) encompass phenotypically distinct clinical diagnoses. To correlate clinical symptoms with the underlying immune mechanism, drug hypersensitivity and other immune reactions are often

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Table 15.1 Revised Gell–Coombs classification of hypersensitivity reactions.

Type of reaction	Antigen	Immune reactant	Effector	Clinical symptoms
Type I (immediate)	Soluble antigen	IgE	Mast cell, basophil	Pruritus, angioedema, urticaria, bronchospasm
Type II (cytotoxic)	Cell- or matrix-associated antigen	IgG	FcR positive cells (phagocytes, NK cells)	Thrombocytopenia, hemolytic anemia
Type III (immune complex)	Soluble antigen	IgG	FcR positive cells, complement	Serum sickness
Type IV (delayed)	Antigen presented by HLA proteins or direct T-cell stimulation	T-cell-mediated		
		IVa	Macrophage	Contact dermatitis
		IVb	Eosinophils	DRESS/DIHS/HSS
		IVc	T cells	SJS/TEN, DILI
		IVd	Neutrophils	AGEP

Ig, immunoglobulin; FcR, Fc receptor; NK cells, natural killer cells; DRESS, drug reaction with eosinophilia and systemic symptoms; DIHS, drug-induced hypersensitivity syndrome; HSS, hypersensitivity syndrome; SJS, Stevens-Johnson syndrome; TEN, toxic epidermal necrolysis; DILI, drug-induced liver injury; AGEP, acute generalized exanthematous pustulosis.
Source: Adapted from Padmanabhan, 2014. Adapted from Rive et al. [13].

classified into four categories described by Coombs and Gell [7, 18]. Traditionally, T-cell-mediated reactions have been classified as Gell–Coombs type IV reactions. This classification system has recently been revised to include four subtypes, each representing the specific effector cell involved. (Table 15.1) [13].

HSRs include conditions such as drug-induced hypersensitivity syndrome (DIHS)/drug reaction with eosinophilia and systemic symptoms (DRESS)/hypersensitivity syndrome (HSS), Stevens–Johnson syndrome/toxic epidermal necrolysis (SJS/TEN), acute generalized exanthematous pustulosis (AGEP), drug-induced lupus, fixed drug eruption, and single-organ disorders like drug-induced liver injury (DILI). With the exception of DILI, where toxic mechanisms may be important, all of these syndromes are thought to be primarily immunologically mediated. Since many of these DHS are linked to severe cutaneous manifestations, the term "severe cutaneous adverse reaction" (SCAR) is commonly used to collectively refer to DIHS/DRESS, SJS/TEN, and AGEP.

15.2.1 DIHS/DRESS/HSS

DRESS is also known as DIHS or HSS. It typically manifests 2–8 weeks after exposure to the causative drug and represents a potentially life-threatening drug

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HSR characterized by varied combinations of skin eruption, fever, facial edema, lymphadenopathy, visceral involvement, and hematologic abnormalities, including eosinophilia and atypical lymphocytosis [19].

It is assumed that DRESS is the result of a complex interaction between drug (or vaccine or biologic) exposure, genetic predisposition, and viral reactivation [19].

Diagnosing acute DIHS/DRESS can be challenging as it shares similar features with infections, lymphoproliferative disorders, and autoimmune diseases. Due to its long latency period, it can be mistaken for other SCARs. However, significant organ involvement, hematological features, and a rash without mucosal involvement distinguish DIHS/DRESS from SJS/TEN and AGEP.

15.2.2 SJS/TEN

SJS and TEN are the most severe cutaneous HSSs with high morbidity and mortality rates. They are characterized by a painful blistering skin rash that can lead to significant epidermal necrosis and detachment, sepsis, and potentially death. These syndromes are often associated with multiorgan involvement (anemia, lymphopenia, complications from epidermal fluid losses, and superimposed bacterial infection/sepsis). Fever is common and patients may experience a sore throat and/or visual impairment. According to the percentage of the involved total body surface area (TBSA), the two syndromes represent a spectrum of the same clinical picture. Generally, blistering and epidermal detachment on <10% of TBSA is diagnosed as SJS. TEN is diagnosed in instances where >30% of TBSA is affected. SJS/TEN describes the overlap in this spectrum, where 10–30% of TBSA is affected [20].

Over 80% of reported cases are linked to medication exposure, with more than 300 different implicated drugs and supplements [21, 22]. Most cases occur 4–28 days after initial exposure to the drug. However, there have also been case reports of delayed-onset SJS/TEN (after six months) due to lamotrigine, as well as rapid-onset SJS/TEN (within three days) caused by acetaminophen and penicillin [7, 23–25].

SJS/TEN represents a severe T-cell-mediated type of HSR. This reaction results from an interaction between a major histocompatibility complex (MHC)-bound peptide on an antigen-presenting cell (APC) and a T cell receptor (TCR) expressed on a $CD8^+$ (cytotoxic) T cell. These drug-reactive T cells may originate from skin resident memory T cells (T_{RM}) and/or circulating $CD8^+$ T cells. Activated $CD8^+$ T cells stimulate the production of downstream cytokines/chemokines and trigger epidermal keratinocyte apoptosis through the Fas/Fas ligand (FasL) pathway and TCR/HLA pathway [7, 26, 27]. Additionally, natural killer (NK) cells and drug-activated monocytes are discussed to trigger keratinocyte apoptosis and necroptosis [28, 29].

Given the crucial role of MHC in the pathogenesis of SJS/TEN and other drug HSRs, many of the genetic variants most strongly associated with the condition are HLA alleles. Other genetic risk factors include polymorphisms in genes required for drug metabolism (e.g. cytochrome P450), in antigen presentation (CYP2C9 and CYP2C19) [30, 31], and in MHC presentation (proteasome pathway) [32, 33].

Because of the overlapping clinical features with other dermatologic diseases such as erythema multiforme, staphylococcal scalded skin syndrome, pemphigus

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vulgaris, and acute graft versus host disease, SJS/TEN can be difficult to identify. As those diseases require different treatments and have distinct prognoses, careful diagnostic procedure is important [7].

15.2.3 Single-Organ Drug Hypersensitivity Syndromes

Isolated hepatic dysfunction has been associated with several drugs including flucloxacillin, lumiracoxib, lapatinib, and ximelagatran [34–36]. This syndrome, termed DILI may manifest as hepatocellular injury or cholestasis (obstruction of normal bile flow) and has been shown to progress to fulminant hepatic failure in rare instances [37]. The mechanisms underlying these pathologies may include both a toxic and an immunological basis [36]. Isolated pancreatitis or tubulointerstitial nephritis are additional examples of drug HSRs with single-organ involvement.

15.3 Immunopathogenesis of Drug Hypersensitivity Syndromes

In recent years, research focused on the link between gene polymorphisms and metabolic or immunological pathways to explain altered drug efficacy and susceptibility to adverse events. Since HLA plays a fundamental role not only in the presentation of self-antigen to T-cells for tolerance but also for foreign antigens for cytotoxic destruction, it is not surprising that specific HLA alleles are risk factors and may predispose to (or protect from) disorders in which the immune system is strongly implicated (e.g. autoimmunity, cancers, infectious diseases) [6, 38].

Given the co-dominant HLA expression, the predisposition to drug hypersensitivity depends on the presence of the relevant hypersensitivity-associated allele. Both heterozygote and homozygous individuals have an increased risk of drug-induced hypersensitivity. On the other hand, the absence of the allele indicates that the patient has a very low risk of HDR associated to a specific drug [6, 39]. However, the presence of an HLA "risk allele" does not always result in the occurrence of an adverse reaction when an individual is exposed to a drug.

15.3.1 Immunopathogenic Models

Immunogenic complexes involved in T-cell-mediated ADRs are formed from three components: HLA, peptide, and drug. However, the peptide ligands of HLA class I (8–12 mers) and class II (9–25 mers) molecules are usually bigger than most drugs, which are more comparable in size to 1–3 amino acids [40]. Therefore, it is not clear how those drugs can initiate a T cell response. The pathway of antigen-processing and presentation possesses several options for small molecules to interact and generate novel HLA–peptide complexes: preprocessing, interference with peptide loading, and cell surface modification. These interactions can be either allotype-specific (due to interaction with a unique site within the HLA molecule), HLA subtype-specific

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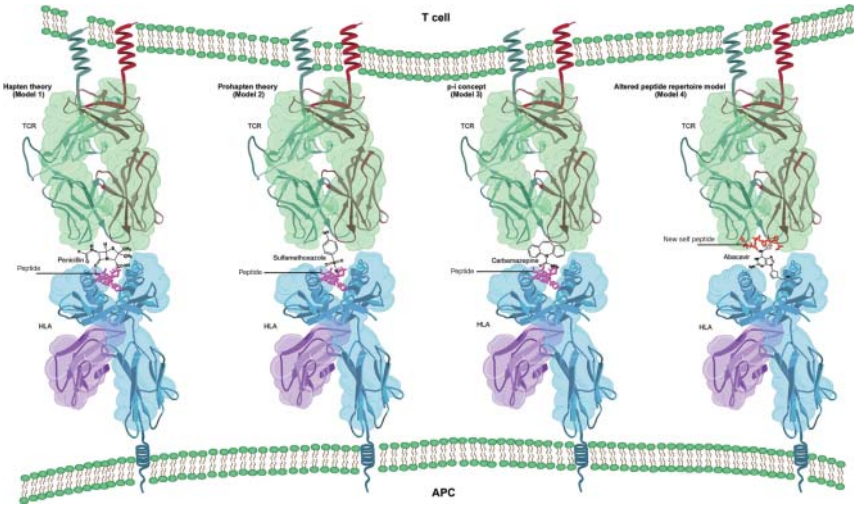

Figure 15.1 Mechanisms of immunopathogenesis in adverse drug reactions (ADRs) are described by four theories of interactions between HLA, drugs, peptides, and T lymphocytes. The Hapten theory (model 1) describes the covalent binding of drugs to endogenous peptides before antigen processing leading to the formation of neo-antigens which are then presented. The Prohapten theory (model 2) postulates that some chemically inert drugs (prohaptens) may be metabolized to reactive molecules that later serve as haptens. The pharmacological interaction (p-i) concept (model 3) describes the reversible non-covalent binding of drugs directly to the HLA molecule, antigen, or T-cell receptors without intracellular processing and the altered peptide repertoire model (model 4) explains how a drug can alter the specificity of the peptide-binding groove through binding the F-pocket leading to the presentation of unconventional self-peptides. APC, antigen-presenting cell; HLA, human leukocyte antigen; TCR, T-cell receptor. Source: [42]/IntechOpen/CC BY 3.0.

(due to interaction with a site conserved across a subgroup of HLA), or promiscuous across diverse HLA [41].

The three classic immunopathogenic models (i) the hapten/prohapten model, (ii) the pharmacological interaction (p-i) with immune receptors concept, and (iii) the altered peptide repertoire hypotheses can be supplemented by (iv) the heterologous immunity model and (v) the danger model. The aforementioned immunopathogenic theories are not mutually exclusive and a specific mechanism may be more prevalent for one drug but not necessarily for another (Figure 15.1) [6].

15.3.1.1 The Hapten/Prohapten Model

The immunologist Karl Landsteiner proposed the hapten/prohapten hypothesis [43]. A hapten is a small low-molecular-weight compound (<1,000 D) that elicits an immune response only when covalently bound to a larger protein, thereby altering its structure or chemical composition. When bound to cellular proteins, they can stimulate the innate immune system by upregulation of CD40 or by binding to TLR7 and TLR8 directly to stimulate dendritic cells [44, 45]. This was shown for the drug imiquimod [46]. Additionally, haptens may stimulate the specific immune system. The hapten-carrier complex undergoes intracellular processing and represents a

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neoantigenic structure that subsequently elicits a T-cell response. Modified proteins can also be immunogenic for B cells, triggering a humoral immune response. This hapten modification can occur with soluble proteins, cell-bound proteins, or with the MHC–peptide complexes themselves, resulting in distinct immune responses.

The hapten/prohapten model also postulates that some chemically inert drugs (prohaptens) may be metabolized to reactive molecules that later serve as haptens [10, 47].

HSRs associated with hapten modification of endogenous proteins are, for example, the binding of penicillin derivatives to serum albumin [48] and the modification of endogenous proteins by the nitroso-sulfamethoxazole metabolite of sulfamethoxazole [49].

15.3.1.2 The p-i Model

The p-i model, an abbreviation for "pharmacological interaction," postulates that some chemically inert drugs may bind specifically and reversibly to some of the highly variable antigen-specific receptors in a direct way, instead of covalently modifying the MHC–peptide complex. The activation of T cells occurs in the absence of a specific peptide ligand.

The p-i model might explain delayed-type HDRs that are observed at the first encounter with a drug without the sensitization phase required for reactions adhering to the hapten/prohapten concept [50]. Examples of DHSs thought to occur via the p-i model include those for lidocaine and lamotrigine.

15.3.1.3 The Altered Peptide Repertoire Model

The altered peptide repertoire model states that a drug noncovalently binds to the peptide-binding groove of an HLA molecule. The structure of these pockets is highly allele-specific, thereby dictating peptide-binding preferences for each HLA molecule. In this model, the drug that is noncovalently bound to the binding groove changes the chemistry and shape of the peptide binding cleft, thereby altering the repertoire of self-peptide ligands that are bound and presented by APCs. The altered peptide repertoire includes novel antigens and can lead to a primary and polyclonal immune response [51–54].

15.3.1.4 The Heterologous Immunity Model

According to this model, cross-reactive TCRs recognize different peptides and stimulate the activation of the immune system. For example, with abacavir reacting T cells also recognize herpes viruses-derived peptides [55]. Additionally, in allergic individuals, the immune system may have been activated prior to drug exposure, leading to the proliferation of activated T cells. [56]. Thus, this model describes the generation of an immune-mediated ADR as an interplay of an HLA risk allele, an infection by a pathogen and the generation of a pathogen-specific memory T cell response that is cross-reactive with the drug [57].

15.3.1.5 The Danger Model

In this model, signals other than the drug itself (chemical, physical, viral stress) help overcome immune tolerance barriers that normally would suppress DHRs

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[58, 59]. This model could explain the observation that drug allergy appears more often in people who have concomitant viral infections. This suggests a potential role of viruses in the development of non-HLA- restricted drug allergy [60].

15.4 HLA and Hypersensitivity Reactions

The genes of the HLA class I complex, HLA-A, HLA-B, and HLA-C genes, are among the most highly polymorphic genes in the human genome. For example, more than 10,000 HLA-B alleles and more than 8,000 HLA-A alleles have been identified in populations worldwide and deposited to the World Health Organization Nomenclature Committee for Factors of the HLA System [61]. Several allelic variants of HLA class I and class II alleles have been associated with hypersensitivity to drugs or ADR, but risk associations for different delayed hypersensitivity reactions (DHRs) remain statistically weighted toward HLA class I and the pure T-cell-mediated reactions.

The most important HLA alleles for pharmacogenetics, with a significant effect on therapeutic outcomes, are *HLA-A*31:01, HLA-B*15:02, HLA-B*57:01* and *HLA-B*58:01*. Since the allelic frequencies differ across populations, the therapeutic role of different alleles varies and *HLA* genotype screening before initiation of the therapy is recommended only in populations with high prevalence.

15.4.1 Abacavir and HLA-B*57:01

15.4.1.1 Abacavir and Abacavir Hypersensitivity Reaction

Abacavir is an antiretroviral medication used primarily in the treatment of HIV type 1 (HIV-1) infection. It is a nucleoside analog reverse-transcriptase inhibitor (NRTI) of virus replication and is usually administered orally in combination with other antiretroviral agents. Abacavir is a deoxy-guanosine base, which is metabolized by the liver into abacavir triphosphate, the active intracellular agent [62]. The drug does not inhibit or induce cytochrome P-450 enzymes and does not interact with medications metabolized by this system [63]. In clinical trials with patients naive to antiretroviral therapy, abacavir in combination with zidovudine and lamivudine has resulted in a strong reduction in plasma HIV RNA level [64–67].

Despite its efficacy, abacavir is associated with a potentially life-threatening HSR. Approximately 5–8% of abacavir-treated patients are affected during the first 6 weeks of antiretroviral therapy [68, 69].

The abacavir hypersensitivity reaction (AHR) is characterized by skin rash, fever, malaise, gastrointestinal and respiratory symptoms, and may escalate to a systemic illness. Rash, which commonly manifests early in many DHS, is absent in up to 30% of patients with AHR but often occurs later in the course of the disease [70]. Severe forms of the skin rash may result in SJS, TEN, or systemic lupus erythematosus [71]. An early diagnosis of AHR remains a diagnostic challenge to clinicians because of the nonspecific combination of symptoms. The symptoms overlap with features of many common infections, reactions to other drugs, or a general inflammatory state

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expected after HIV-1 treatment initiation. If a patient experiences an HSR immediate discontinuation of abacavir therapy is mandatory [72–74].

Rapid return of symptoms, which are often more severe and potentially life-threatening, can be observed after restarting abacavir therapy. Therefore, restarting the abacavir therapy is contraindicated [75–77].

15.4.1.2 Association with HLA-B*57:01

The link between abacavir and HLA-B*57:01 is one of the most well-established pharmacogenetic associations. Research has shown that individuals carrying the HLA-B*57:01 allele are at a significantly increased risk of developing AHR. It was shown that abacavir can alter the binding capacities of this HLA molecule by creating extensive, noncovalent contacts to residues within multiple binding pockets (C, D, E, and F pockets). This prohibits peptide binding at these sites. Although peptides were shown to maintain contact at the preferential anchor site in the B pocket of the binding cleft, these bound peptides make very few contacts with the drug itself [51, 54].

By interaction with these multiple sites of the HLA-B*57:01 molecule, abacavir changes the chemistry and topography of the peptide binding groove. These changes result in a significantly altered peptide presentation: 20–25% of the peptides bound by the HLA molecule after abacavir administration differed from those that bound before treatment. The novel self-peptides ("altered self" repertoire of peptides) present a new "immunological self" to host T cells and elicit a polyclonal T-cell response that clinically manifests as AHR [54].

These observations of the altered binding capacities and peptide representation are restricted to HLA-B*57:01 and could not be observed with closely related molecules (like HLA-B*57:03), which are not linked to AHR [51]. Closely related allotypes like HLA-B*58:01, HLA-B*57:02, and B*57:03 differ at key residues in the binding cleft. These polymorphisms prevent abacavir binding, and thus peptide selection of these molecules remains unchanged in the presence of the drug.

Given that only about 50% of HLA-B*57:01 positive individuals develop AHR, it is obvious that additional factors determine whether abacavir treatment induces AHR [78]. Whether these cofactors are genetic [79] or are related to environmental factors is currently under investigation.

15.4.1.3 Clinical Implications

The most effective pharmacotherapy for HIV treatment is combination antiretroviral therapy [78]. Minimizing adverse effects is critical to controlling the infection and maintaining the treatment. Therefore, screening for HLA-B*57:01 before initiating abacavir therapy has become standard practice in many countries. Patients positive for HLA-B*57:01 are advised against using abacavir to prevent AHR, which has been reported to be cost-effective [80, 81]. By comparing randomized individuals to receive HLA-B*57:01 screening versus standard of care, it was shown that HLA-B*57:01 is associated with a 55% positive predictive value (PPV) and a 100% negative predictive value (NPV) for AHR [82]. Importantly, the PPV and NPV were

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consistent across diverse populations [83]. This strategy successfully eliminated AHR and false-positive clinical diagnoses.

15.4.2 Allopurinol and HLA-B*58:01

15.4.2.1 Overview of Allopurinol

Allopurinol is the most widely used urate-lowering medication worldwide. It is a xanthine oxidase inhibitor used to treat hyperuricemia, an excess of uric acid in the body defined as an increase above the reference levels. High levels of uric acid can lead to the deposition of sodium urate crystals in the joints (gout), leading to inflammation and, as a consequence, pain and limited mobility. An increase in uric acid (UA) in the blood enhances its filtration rate by the kidneys, thereby increasing the risk of urate deposits, which can trigger urolithiasis. Additionally, hyperuricemia is a recognized risk factor for the onset and progression of cardiovascular disease [84]. Allopurinol has a half-life of 1–3 hours. It is metabolized in the liver to oxypurinol, the active metabolite with a half-life of 12–17 hours, and is excreted renally [7, 85, 86]. A decline in kidney function decreases the oxypurinol clearance resulting in higher plasma concentrations. Therefore, impaired kidney function represents a risk factor for poor outcomes in AHS. While generally well tolerated, allopurinol therapy can lead to allopurinol hypersensitivity syndrome (AHS), which has a mortality rate of 20–25% [7]. This HSR to allopurinol is characterized by several clinical features, including vasculitis, hepatocellular injury, acute kidney injury, fever, leukocytosis, eosinophilia, and both SJS and TEN [87]. Higher doses of allopurinol and concurrently taking colchicine or statins with allopurinol seem to be risk factors [7, 88]. Other studies indicate that thiazide diuretics, which affect the kidney's ability to process sodium and urate, also increase the risk of severe AHS [89]. In addition, genetics contribute to severe reactions to allopurinol [7].

15.4.2.2 HLA-B*58:01 Association

The HLA-B*58:01- allele is most prevalent in Asian subpopulations, particularly in individuals of Korean, Han-Chinese, or Thai descent (Figure 15.2). In 1989, the first report of a potential genetic association between allopurinol administration and observed ADRs occurred in Southern Chinese patients with cutaneous manifestations of allopurinol hypersensitivity [86, 92]. A case-controlled genome-wide association studies (GWAS) identifying the HLA-B*58:01 genotype as strongly associated with allopurinol-induced SJS/TEN in the Han Chinese population followed. This study showed the presence of the HLA-B*58:01 allele in 100% of cases of allopurinol-SJS/TEN/DRESS/DIHS/HSS, in 15% of allopurinol-tolerant patients and in 20% of healthy controls [86, 93]. However, an *in vitro* study has shown that the HSR to this drug is mediated by a T cell response specific to oxypurinol, with oxypurinol concentration as a key factor in T cell sensitization [94]. Furthermore, this response was more pronounced in those individuals with HLA-B*58:01 allele [94]. The rapid binding of oxypurinol to HLA-B*58:01 takes place on the cell surface by interacting with the peptide-binding groove of the HLA-B*58:01 molecule. Peptide processing is not required. The novel drug peptide–HLA complex

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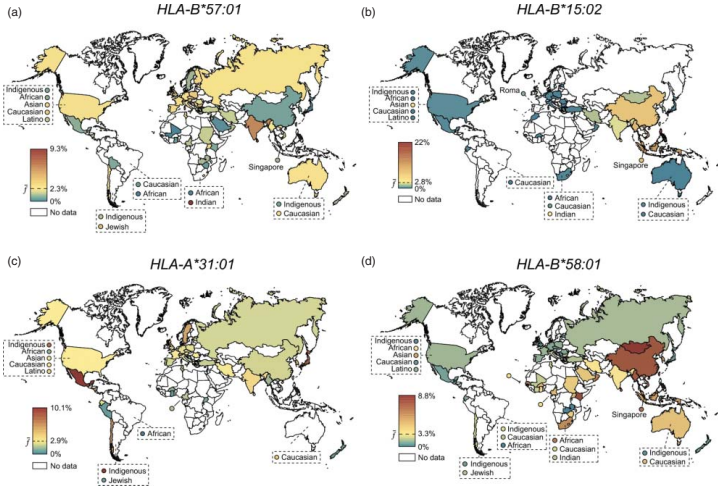

Figure 15.2 Global distribution of clinically important human leukocyte antigen (HLA) alleles. Allele frequencies of HLA-B*57:01 (a), HLA-B*15:02 (b), HLA-A*31:01 (c), and HLA-B*58:01 (d) across up to 74 countries are shown. Countries are color-coded with the highest frequency in red, the average frequency across all populations ($\bar{f}$) in yellow, and the lowest frequency in blue. Countries for which no HLA frequency information was available are colored white. Figure modified with permission from [90, 91].

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elicits a polyclonal T-cell response resulting in allopurinol-related SCARs. Presumably, the direct binding depends on both dose and binding affinity. The presence of HLA-B*58:01 and higher oxypurinol concentrations would act synergistically to increase the risk of T cell sensitization [86, 88].

15.4.2.3 Clinical Implications

Given the strong association of HLA-B*58:01 with allopurinol-induced SCARs, a screening for HLA-B*58:01 is recommended in high-risk populations before initiating allopurinol therapy. Patients who test positive should avoid allopurinol and consider alternative treatments for hyperuricemia. If there is no alternative, for patients who carry HLA-B*58:01 and suffer from chronic kidney disease (CKD) a tolerance induction protocol should be applied when commencing allopurinol. This can reduce the risk of developing allopurinol-related SCAR [86, 95].

15.4.3 Carbamazepine and HLA-B*15:02/HLA-A*31:01

15.4.3.1 Overview of Carbamazepine

Carbamazepine is an effective anticonvulsant and mood-stabilizing drug that is used not only in the treatment of epilepsy and other psychiatric disorders [96, 97] but as an effective treatment in trigeminal neuralgia and bipolar disorder [98]. Carbamazepine is a tricyclic compound that is metabolized in the liver by the cytochrome P-450 (CYP) system and blocks voltage-dependent sodium channels present on neuronal cell membranes. The inactivated state of these sodium channels is stabilized and a smaller amount is available to open. This prolonged inactivation of the channel inhibits a rapid and repetitive generation of action potentials in the epileptic focus [7].

HSRs to carbamazepine can occur in up to 10% of patients and typically appear 2–8 weeks after initiation of the drug. These reactions may include rash, fever, lymphadenopathy, and internal organ involvement [99].

Several studies found strong associations of not only HLA-B*15:02, but also with the similar HLA-B75 serotypes (e.g. HLA-B*15:11, HLA-B*15:21) and HLA-A*31:01 with CBZ-induced cutaneous ADRs (e.g. SJS, TEN, MPE, or DRESS) [100, 101]. The molecular mechanism is not fully understood, yet. The clinical presentation of the CBZ-mediated ADRs differs for HLA-B*15:02 and HLA-A*31:01. HLA-B*15:02 is linked to severe SJS and TEN, whereas carriers of HLA-A*31:01 present with milder symptoms including MPE and DRESS [102, 103].

15.4.3.2 HLA-B*15:02 Association

CBZ-induced SJS/TEN has been strongly associated with carriage of HLA-B*15:02 in patients from South East Asia and results from the higher prevalence of this allele in those countries [101, 102, 104, 105]. Allele frequencies are particularly high in the Philippines (22%), Vietnam (13.8%), Indonesia (11.6%), and Malaysia (11.5%), with the notable exception of Japan (< 0.1%) [90]. A prospective cohort study in Taiwan demonstrated the clinical utility of pharmacogenetic screening for HLA-B*15:02 in preventing CBZ-induced SJS/TEN [106]. Therefore, regulatory agencies, like the US

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Food and Drug Administration (FDA) and the European Medicines Agency (EMA), advise pharmacogenetic screening in patients from particular populations in South East Asia [107].

Carbamazepine HSRs are not present in all HLA-B*15:02 carriers. One additional factor contributing to the susceptibility is specific TCR clonotypes, like VB-11-ISGSY and VB-11-GLAGVDN. These TCR clonotypes are present in different allergic HLA-B*15:02 patients but absent from carbamazepine-tolerant HLA-B*15:02 subjects. These studies imply, that, in the case of HLA-B*15:02-restricted carbamazepine-SJS/TEN, the presence of both the HLA-B*15:02 allotype and a specific TCR clonotype is predictive of the development of carbamazepine-induced ADR [108]. Since only 84% of clinical samples presented with this dominant TCR clonotype, there seem to be other contributing factors.

Recent investigations found that the main metabolite EPX (CBZ-10, 11-epoxide) triggers B*15:02-associated CBZ hypersensitivity. EPX had a greater effect on the peptide-binding motif than CBZ [42, 103, 109] and decreased the presentation of 9-mers by HLA-B*15:02 [103]. Furthermore, the level of EPX seems to have a bigger influence than the initial dose of CBZ, since the occurrence of an ADR was independent of the CBZ dosage [110]. These facts imply that not only the HLA allele and TCR but also drug metabolism plays a role in the development of this HSR.

15.4.3.3 HLA-A*31:01 Association

In addition to HLA-B*15:02, the HLA-A*31:01 allele has been implicated in carbamazepine-induced ADRs. In contrast to patients positive for HLA-B*15:02 CBZ HSRs in these patients seem to be associated more frequently with DRESS/DIHS/HSS and maculopapular exanthema (MPE) [111, 112].

The HLA-A*31:01 association is common worldwide, suggesting a broader relevance. It is most prevalent in indigenous populations in the Americas, such as in Argentina (28.8%), Mexico (10.1%), the United States (7.8%), Nicaragua (6.7%), and Chile (6.6%), whereas frequencies in Africa and Oceania seem to be lower (approximately 1%) [90].

The molecular mechanism in HLA-A*31:01-positive patients is not fully understood. It is possible that alterations of the proteome including increased ubiquitination play a role in the reaction. Furthermore, the binding of CBZ to the HLA heavy chain, the simultaneous presentation of unmodified low-binding peptides, and the altered presentation of high-binding peptides might affect the accessible surface for a TCR and potentially the half-life time of the peptide–HLA complexes [113].

15.4.3.4 Clinical Implication

Since 2007, when the FDA added a warning to the CBZ drug label regarding the risk of carbamazepine-induced SJS/TEN, a screening for HLA-B*15:02 is recommended in South-East Asian populations [7, 114]. Carbamazepine should be avoided in patients who carry the HLA-B*15:02 variant.

It is important to notice that up to 80% of patients with an unexpected CBZ HSR will also have an adverse reaction to other antiseizure drugs. This risk of cross-reactivity between structurally similar antiseizure drugs (oxcarbazepine,

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lamotrigine, phenytoin, phenobarbital, primidone) limits treatment options and demands a cautious surveillance [115–118].

15.4.4 HLA Associations in DILI

15.4.4.1 Overview of DILI

DILI is one of the leading causes of acute liver failure and is associated with high morbidity and mortality.

Since severe drug reactions mimic other etiologies of liver damage or renal impairment, the diagnosis and the association to drug treatment are sometimes difficult. DILI can be classified into an allergic and non-allergic form. Allergic DILI is often related to HLA genetic factor and results in abnormal immune response. Non-allergic DILI, on the other hand, is the result of the accumulation of related reagents within the liver [119].

The association of genetic factors with DILI has been known since the 1980s, but it was only after the development of GWAS that specific genetic risk factors could be detected and confirmed [120]. It is important to note, that there is no specific HLA allele associated with the general risk for DILI. While HLA alleles are drug-specific, DILI risk for a particular drug may extend across several HLA alleles, such as those with shared peptide specificities. Additionally, one specific HLA risk allele may be linked to different drug reactions with varying phenotypes and very different clinical implications, as in the case of HLA-B*57:01 with flucloxacillin DILI and abacavir HSS.

15.4.4.2 Amoxicillin and Clavulanate-Associated DILI

The combination of amoxicillin and the beta-lactamase inhibitor clavulanic acid is one of the most frequently prescribed antimicrobials for the treatment of bacterial infections. The average onset of symptoms from first starting amoxicillin/clavulanate treatment is 18 days. These patients often present with features associated with hypersensitivity including rash, fever, and eosinophilia. The majority of patients make a full recovery within 4–6 months, but a few patients develop fulminant hepatic failure and require transplantation.

The precise pathophysiological mechanisms underlying the phenotype–genotype association in AC-DILI are still unknown. Research suggests that liver injury is primarily caused by an immune-mediated attack on cholangiocytes or perhaps on the apical pole of hepatocytes. The specific antigens, which induce the cellular immune response, remain currently unknown. It seems likely that neo-antigens, produced by the beta-lactam structures of amoxicillin or clavulanic acid, react with the proteins of the susceptible patients. Neo-antigens might be produced in many people who take AC, but only a small group with specific genetic susceptibilities may present with DILI [6, 121].

The association of HLA with amoxicillin/clavulanate and DILI was first established in 1999 when a study reported a significantly increased frequency of the HLA haplotype DRB1*15:01-DRB5*01:01-DQB1*06:02 in a European population of patients with amoxicillin/clavulanate-induced DILI compared to control subjects

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(57.1% compared to 11.1%) [122]. The association between HLA-DRB1*15:01 and amoxicillin/clavulanate-induced DILI has been replicated by a number of studies, and in recent years, additional specific haplotypes have been associated. Cholestatic DILI has been associated with the DRB1*15:01/DQB1*06:02 haplotype whilst HLA-A*30:02/HLA- B*18:01 has been linked with hepatocellular DILI [123].

Taken together, different HLA risk alleles of class I and class II are associated with phenotypically different presentations of AC-DILI.

15.4.4.3 Flucloxacillin-Associated DILI

Flucloxacillin is a beta-lactam antibiotic used for the treatment of staphylococcal infections and shows the strongest HLA association for DILI [124]. It is associated not only with HLA-B*57:01 but also HLA-B*57:03, which share significant peptide binding specificities because they only differ by 2 amino acids [124]. HLA-B*57:03 is rare among Northern Europeans (estimated allele frequencies of 0 (Northern Ireland), 0.002 (Irish Republic), and 0.0007 (Germany; www.allelefrequencies.net)). Only ~1 in every 500 HLA-B*57:01 carriers are likely to develop DILI if treated with flucloxacillin. Therefore, this test is not routinely used prior to the prescription of the drug [125].

Mechanisms of Adverse Reactions Flucloxacillin covalently binds to selected lysine residues on circulating albumin. The degree of binding controls the intensity of the T cell proliferative response and subsequent immune reaction [126]. In addition, flucloxacillin-specific mononuclear cell responses were found together with flucloxacillin reactive $CD4^+$ and $CD8^+$ T cell clones in peripheral blood samples taken from individuals with a history of DILI. Furthermore, flucloxacillin is able to activate naive $CD8^+$ T cells in HLA-B*57:01 positive individuals [127].

At present, because of low NPV and PPV, there is no clinical use for screening of HLA risk alleles prior to medication with high-risk DILI medications. In cases where the NPV is 100%, meaning that the specific HLA risk allele is necessary but not sufficient, the number needed to test is prohibitively high. The number of patients that is needed to test is influenced by the PPV, the frequency of the risk allele in the target population, and the prevalence of the DILI reaction. When implementing an HLA risk allele screening also other important factors, like cost-effectiveness, ease of screening in clinical practice, the economic consequences of the disease, and the availability of alternative drugs with a better safety profile or without an associated genetic risk, have to be considered [128].

15.4.5 Other HLA Associations with Drug Hypersensitivity Syndromes

The increasing interest in pharmacogenomics and advanced research methods allow the identification of new relations and correlations between a growing number of specific HLA alleles and drug-induced HSRs. These associations are usually drug and population-specific (Table 15.2, [129]). Only a subset of specific HLA molecules may have higher binding affinities for specific drug antigens and present the drug antigens to specific TCRs, causing a series of T cell activations

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Table 15.2 HLA association with various phenotypes of drug hypersensitivity in different populations.

Associated drug	HLA allele	Hypersensitivity reactions	Ethnicity
Aromatic anticonvulsants			
Carbamazepine	*B*15:02*	SJS/TEN	Han Chinese, Thai, Indian, Malaysian, Vietnamese, Singaporean, Hong Kongese
	*A*31:01*	DRESS	Han Chinese, European, Spanish
	*A*31:01*	DRESS/SJS/TEN	Northern European, Japanese, Korean
	*B*15:11*	SJS/TEN	Han Chinese, Japanese, Korean
	*B*59:01*	SJS/TEN	Japanese
	*B*38:01*	SJS/TEN	Spanish
	*B*15:02*	SJS/TEN	Han Chinese, Thai
	*B*15:02*	SJS/TEN	Han Chinese, Thai
Oxcarbazepine Phenytoin	*B*15:02, B*13:01, B*51:01*	SJS/TEN	Han Chinese, Japanese, Malaysian
	*A*33:03, B*38:02, B*51:01, B*56:02, B*58:01, C*14:02*	SJS/TEN	Thai
	*B*51:01*	DRESS	Thai
	*B*15:13*	DRESS/SJS/TEN	Malaysian
	*CYP2C9*3*	DRESS/SJS/TEN	Han Chinese, Japanese, Malaysian
	*CYP2C9*3*	SJS/TEN	Thai

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Phenobarbital Lamotrigine	*B*15:02*	SJS/TEN	Han Chinese
	*B*38; B*58:01, A*68:01, Cw*07:18*	SJS/TEN	European
	*B*38:01*	SJS/TEN	Spanish
	*A*31:01*	SJS/TEN	Korean
	*A*24:02*	DRESS/SJS/TEN	Spanish
Allopurinol	*B*58:01*	DRESS/SJS/TEN	Han Chinese, Thai, Japanese, Korean, European
Antiretroviral drugs			
Abacavir	*B*57:01*	HSS	European, African
Nevirapine	*DRB1*01:01*	DRESS	Australian
	*B*35:05*	DRESS	Thai
	*B*14:02, Cw*08:01, Cw*08:02*	HSS	Sardinian, Japanese
	*C*04:01*	DRESS/SJS/TEN	Malawian
Antibiotics			
Beta-lactam	*DR9, DR14.1, DR17, DR4*	Immediate-type drug hypersensitivity	Chinese
	DRA rs7192, *DRA* rs8084	Immediate-type drug hypersensitivity	Spanish, Italian
Cotrimoxazole	*B*15:02, C*06:02, C*08:01*	SJS/TEN	Thai
Dapsone	*B*13:01*	HSS	Han Chinese

(Continued)

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Table 15.2 (Continued)

Associated drug	HLA allele	Hypersensitivity reactions	Ethnicity
Sulfamethoxazole	*B*38:02*	SJS/TEN	European
Sulfonamide	*A*29, B*12, DR*7*	TEN	European
NSAIDs			
Aspirin	*DRB1*13:02, DRB1*06:09*	Urticaria/angioedema	Korean
Aspirin and other NSAIDs	*DRB1*11*	Urticaria/angioedema and hypotension/laryngeal edema	Spanish
Aspirin and other NSAIDs	*B*44, Cw*5*	Chronic idiopathic urticaria	Italian
Oxicam NSAIDs	*B*73:01*	SJS/TEN	European
Other drugs			
Methazolamide	*B*59:01, CW*01:02*	SJS/TEN	Korean, Japanese

The mentioned associations are specific for the dedicated populations.
DRESS, drug reaction with eosinophilia and systemic symptoms; HSS, hypersensitivity syndromes; MPE, maculopapular exanthema; NSAIDS, nonsteroidal anti-inflammatory drugs; SJS, Stevens–Johnson syndrome; TEN, toxic epidermal necrolysis.
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and adverse immune responses [129]. This affects only a small subset of patients; therefore, research related to these associations is sparse. Table 15.2 includes HLA alleles specific to small populations.

15.4.6 Pharmacogenetic Important HLA P Groups

P and G Codes are used to simplify the reporting of ambiguous allele typings. These codes are defined in the HLA nomenclature (source http://hla.alleles.org). HLA alleles that are described by a G group have identical nucleotide sequences across the exons encoding the peptide binding domains (exon 2 and 3 for HLA class I and exon 2 only for HLA class II alleles). The alleles combined in one P group can differ in the nucleotide sequence but have identical amino acid sequences encoded by exon 2 and 3 for HLA class I and exon 2 only for HLA class II alleles. Thus the alleles combined in HLA P groups encode for proteins with identical protein sequences of the antigen binding sites, which represent the site of drug interaction proposed in all models. This implies that those HLA proteins will act similarly in the way of antigen binding and are therefore just as important for drug interactions as the associated alleles. This should be kept in mind in diagnostics (Table 15.3).

Table 15.3 Pharmacogenetic important HLA P groups; assigned as of June 2024.

HLA P group	HLA allels of this group
A*31:01P	*A*31:01, A*31:23, A*31:46, A*31:48, A*31:55, A*31:56, A*31:59, A*31:71, A*31:72, A*31:81, A*31:95, A*31:111, A*31:119, A*31:125, A*31:128, A*31:132, A*31:135, A*31:143, A*31:151Q, A*31:152, A*31:153, A*31:155, A*31:156, A*31:157, A*31:159, A*31:160, A*31:166, A*31:167, A*31:177, A*31:178, A*31:181, A*31:182, A*31:186, A*31:191, A*31:194, A*31:196, A*31:197, A*31:198, A*31:199, A*31:208, A*31:209, A*31:212, A*31:215, A*31:224, A*31:226, A*31:227, A*31:229, A*31:233*
B*15:02P	*B*15:02, B*15:214, B*15:358, B*15:437, B*15:537, B*15:557, B*15:630, B*15:664, B*15:681*
B*57:01P	*B*57:01, B*57:29, B*57:37, B*57:55, B*57:91, B*57:103, B*57:116, B*57:132, B*57:135, B*57:136, B*57:138, B*57:144, B*57:158, B*57:159, B*57:163, B*57:164, B*57:166, B*57:168, B*57:169, B*57:171, B*57:173, B*57:184*
B*58:01P	*B*58:01, B*58:11, B*58:51, B*58:52, B*58:57, B*58:58, B*58:66, B*58:71, B*58:95, B*58:99, B*58:101, B*58:103, B*58:105, B*58:106, B*58:108, B*58:112, B*58:113, B*58:117, B*58:120, B*58:129, B*58:139, B*58:140, B*58:149, B*58:155Q*

15.5 Clinical Implications of HLA Testing

15.5.1 Importance of Pre-Prescription HLA Testing

Pre-prescription HLA testing is a powerful tool in preventing severe drug reactions. By identifying individuals who carry high-risk HLA alleles, healthcare

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providers can make informed decisions about drug therapy, potentially avoiding life-threatening reactions.

Several case studies highlight the success of HLA testing in clinical practice. For example, routine HLA-B*57:01 testing has dramatically reduced the incidence of Abacavir hypersensitivity, while HLA-B*15:02 testing has similarly decreased the rate of Carbamazepine-induced SJS/TEN in at-risk populations.

15.5.2 Challenges

Although specific HLA alleles linked to increased susceptibility to certain drug reactions have been identified, the predictive value of these genetic markers in clinical practice remains unclear. The clinical manifestation of those drug HSRs is influenced by a complex interaction of genetic predisposition, environmental factors, and individual immune responses, which makes it challenging to predict and prevent adverse reactions based only on HLA genotyping. Guidelines by pharmacogenomic expert working groups, such as the Clinical Pharmacogenetics Implementation Consortium (CPIC) and the Dutch Pharmacogenetics Working Group (DPWG) can help to guide drug selection or dosing decisions.

Databases are helpful tools for the prediction of increased susceptibility reactions. For the study of HLA and its role in DHRs, there are some useful databases available, including the Allele Frequency Net Database [130] and the Human Leukocyte Antigen-Adverse Drug Reaction database [131, 132]. Moreover, some easy-to-use *in silico* methods exist that are available via web resources. One example is the Immune Epitope Database (IEDB, www.immuneepitope.org), which predicts the interactions of specific peptides with HLA molecules. This information can be helpful for preliminary immunogenicity assessments and T cell epitope mapping in the early stages of drug development [133]. These tools should be implemented in the development of screening and diagnosing tools in the clinical arena and also applied during drug discovery.

Another challenge remains the identification of allelic variation in non-HLA genetic risk factors, like polymorphic immune loci and killer cell immunoglobulin-like receptors, which are epistatically linked to HLA. These possibly modifying variants could have an effect on HLA-drug interaction and may not be captured on GWAS. By integrating multiple polymorphic immune loci, it may be possible to improve PPV adequately and bring down the necessary number of tests to a clinically useful range.

Since the prevalence of HLA alleles varies between populations, some minor alleles are underrepresented, and it can be difficult to identify new associations. Therefore, those association studies should be performed in diverse populations to ensure their generalizability and clinical applicability.

15.5.3 Ethical Considerations in Personalized Medicine

While HLA testing offers significant benefits, it also raises ethical considerations. Issues such as genetic privacy, the potential for discrimination, and the cost of testing must be carefully managed to ensure equitable access to personalized medicine.

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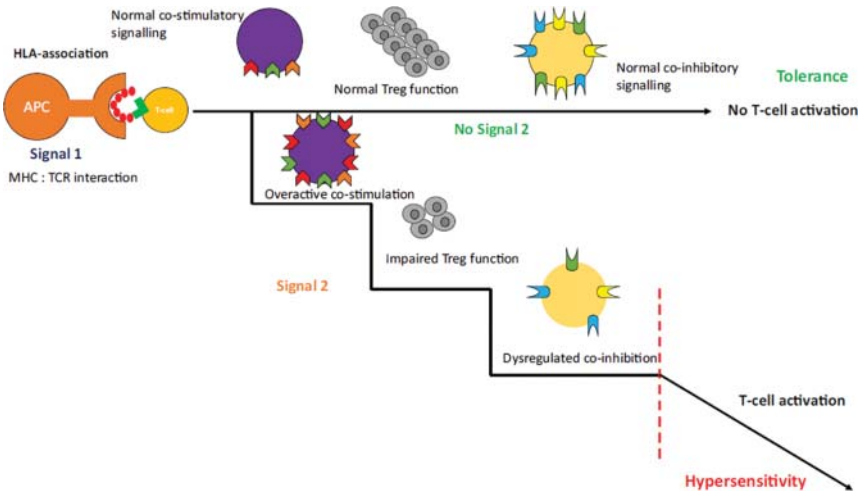

Figure 15.3 Factors leading to hypersensitivity reactions. Source: [134]/John wiley & Sons/CC BY 4.0.

15.6 Future Directions

The field of pharmacogenomics is rapidly evolving, and ongoing research continues to uncover new HLA-drug associations. Current knowledge only represents scratching the surface into this field and other yet-to-be-uncovered factors play a governing role in these reactions. These include genetic factors (HLA expression, TCR repertoire) and immunological regulatory factors (co-inhibition, co-stimulation, regulatory T cell function). Furthermore, environmental factors or the disease status of the host may influence additionally the susceptibility to adverse events. It is likely that a complex interplay between all factors decides between HSRs or tolerance (Figure 15.3; [134]).

As our understanding of drug associations and interactions grows, there is potential for expanding the use of HLA screening to improve drug safety further. Additionally, GWAS and advances in genomic technologies, such as new next-generation sequencing techniques, may enable more comprehensive and cost-effective screening strategies in the future. Those technical advances could pave the way for establishing polygenic risk scores (PRSs) for drug response and safety predictions. PRSs are integrative tools that combine the effects of multiple different genetic variants into a single metric. This score allows the prediction of a given phenotype and the inference of the genetic overlap between traits [2].

15.7 Conclusion

The association between HLA alleles and drug hypersensitivity is a prime example of how genetic factors can influence drug response. Understanding these associations

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is crucial for the safe and effective use of medications. While challenges remain in implementing widespread HLA screening, the benefits of personalized medicine in reducing ADRs and improving patient outcomes are undeniable. With advancing research, the integration of pharmacogenomics into clinical practice will hopefully become routine, paving the way for safer and more tailored therapeutic approaches.

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16

Diseases in the Field of Transfusion Medicine

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16.1 Introduction

The human leukocyte antigen (HLA) system is involved in some transfusion-related adverse reactions, affecting patients receiving blood-derived products, and in conditions or diseases that are recognized to be secondary to alloimmunization mainly due to prior transfusion, pregnancies, or transplantation. Besides HLA, also the human platelet antigen (HPA) and the human neutrophil antigen (HNA) systems are involved; these are specifically addressed in Chapters 4 and 6.

In this chapter, the most relevant diseases and conditions related to the field of transfusion medicine are presented, for which the Laboratory of Histocompatibility and Immunogenetics is asked to support the clinical counterparts in the diagnosis and treatment, including the preventive measures adopted to mitigate the risk of occurrence or to improve the clinical outcomes. Specific knowledge and expertise are required from the Laboratory of Histocompatibility and Immunogenetics to provide helpful support, as well as the awareness of the role played by the abovementioned antigen systems in the pathophysiology of such diseases. An overview of platelet refractoriness, transfusion-related adverse events (i.e. febrile nonhemolytic transfusion reaction, transfusion-related acute lung injury, transfusion-associated graft-versus-host disease, and post-transfusion purpura), neonatal alloimmune neutropenia, and fetal/neonatal alloimmune thrombocytopenia is provided here, giving to the reader the most relevant elements of epidemiology, pathogenesis, clinical features, and therapy or preventive measures.

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16.2 Platelet Transfusion Refractoriness

16.2.1 Definition

Platelet transfusion is essential for preventing thrombocytopenia and hemorrhagic manifestations in patients with hematological and oncological diseases; platelet transfusions have, indeed, been demonstrated to effectively reduce the incidence of severe hemorrhagic complications. However, repeated transfusions are at risk for platelet transfusion refractoriness (PTR) and about 19% of patients develop this condition. PTR is defined as an unsatisfactory post-transfusion platelet count increment after two or more consecutive transfusions of adequate doses of ABO-matched platelets. Patients with hematological malignancies or solid tumors who undergo frequent platelet transfusions are at a higher risk of developing PTR. Studies globally have reported that despite utilizing leukocyte-reduced blood products, PTR persists in about 5–7% of patients with aplastic anemia and hematological malignancies. PTR can induce a series of adverse events, such as spontaneous or life-threatening bleeding, prolonged hospital stays, and increased medical costs. It is an urgent clinical condition that worsens the patient's clinical status and limits the blood resource availability [1–4].

16.2.2 Incidence

The exact incidence of PTR is unknown. Before the widespread use of leukoreduction, PTR was seen in 30–70% of patients with bone marrow failure. However, a Canadian study found that leukoreduction lowered HLA alloimmunization from 19% to 7% and alloimmune PTR from 14% to 4% in patients undergoing chemotherapy for acute leukemia or hematopoietic stem cell transplantation (HSCT). Despite this reduction, PTR remains a significant clinical problem in HSCT and for patients with hematological disorders. Some studies showed that women were more frequently refractory to platelet transfusions than men. PTR tends to occur in parous women, patients with extra-medullary leukemia infiltration, leukopenia, infection, or hemophagocytic syndrome. A retrospective cohort study conducted in the intensive care unit (ICU) found that patients admitted for hepatology/liver transplant had the highest rate of PTR, and larger spleen size was associated with this condition. PTR is often multifactorial and can be due to non-immune and immune causes. About two-thirds of refractory cases are classified as non-immune PTR. The remaining cases are classified as immune PTR, and alloimmunization can occur through previous transfusion, pregnancy, or transplantation. Antibodies against any HLA antigens are detected in 80–90% of cases, followed by antibodies to HPAs; CD36 isoantigen or isoagglutinin to ABO antigens is very rare [5, 6].

16.2.3 Pathogenesis

Platelet refractoriness can be caused by immune and nonimmune factors (Table 16.1).

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Table 16.1 Immune and nonimmune causes of platelet refractoriness.

Nonimmune causes	Immune-mediated causes
• Fever, infection, or sepsis • Bleeding • Accelerated platelet consumption (diffuse intravascular coagulation, microangiopathic hemolytic anemia) • Drugs (amphotericin B, vancomycin, antithymoglobulin, interferons) • Splenic sequestration • GvHD • Poor platelet quality or greater storage age	• Antibodies against HLA class I • ABO-mismatched platelets • Antibodies against human platelet antigens • Antibodies against drug–platelet glycoprotein complex

16.2.3.1 Immunological Causes

Anti-HLA antibodies are the leading factors involved, followed by anti-HPA antibodies and combinations of the two. It has been suggested that minor histocompatibility antigens may be involved in post-transfusion refractoriness, but findings are equivocal and these antigens are generally ignored in the clinical management of refractoriness. When platelets are transfused into a recipient, the recipient becomes exposed to allogeneic donor HLA molecules on the platelet surface. Multiple transfusions and pregnancies increase allogeneic antigen exposure, and once a patient generates HLA alloantibodies, an unsatisfactory platelet count increment follows the platelet transfusion. Anti-HLA antibodies can induce platelet activation and phagocytosis by macrophages; they are also capable of inducing complement activation on the platelets with a membrane attack complex, platelet activation, and subsequent clearance. However, the presence of alloantibodies does not equate to the development of PTR, with some studies showing that a significant number of patients generated alloantibodies after transfusion but did not become refractory. At the same time, higher levels of anti-HLA antibodies were associated with refractoriness in patients with positive lymphocytotoxicity tests, while weak-to-moderate anti-HLA antibody levels detectable by modern binding assays are not associated with PTR. This discrepancy indicates that the immunological PTR mechanism is complex and potentially multifactorial. It may be worth exploring whether genetic variation plays a part in HLA alloimmunization in the context of platelet transfusion. This finding also implies that larger-scale genetic research regarding which variants are associated with platelet-derived alloimmunization is needed. Some patients have highly varied levels of IgG-Fc glycosylation patterns, which may cause different levels of complement activation and clearance. Finally, HLA expression on platelets is highly variable, and different anti-HLA antibody combinations have markedly different effects on the platelets.

Some authors have shown that $CD8^+$ T cells could also play a role in immune-mediated platelet clearance, sometimes in the absence of targeted alloantibodies. There are complex interactions between $CD8^+$ T cells and alloantibodies. Further investigations are needed to explore the relationship between $CD8^+$ T cells

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and antibodies, as well as the process by which $CD8^+$ T cells eliminate allogeneic platelets. Antibodies against HLA are the primary elicitors of immune-mediated PTR; however, other uncommon antibodies should be suspected, such as antibodies to HPA and CD36. Similar to anti-HLA antibodies, anti-HPA antibodies can activate platelets through an FcγR-dependent or complement-activated channel, leading to the rapid removal of foreign platelets. CD36 deficiency is responsible for CD36 isoimmunizations following pregnancy, causing fetal and neonatal alloimmune thrombocytopenia (FNAIT) and sometimes early fetal loss or platelet transfusion, inducing PTR [7–11].

16.2.3.2 Nonimmunological Causes

The systemic inflammatory response and sepsis may prompt platelet consumption and thrombocytopenia through disseminated intravascular coagulation. Interactions between platelets and pathogens, neutrophils and neutrophil extracellular traps, histone proteins, and the activated endothelium have been identified as factors that could directly or indirectly reflect pathophysiologic processes that lead to increased platelet consumption in patient cohorts with exposure to chemotherapy drugs and amphotericin and those with bleeding, fever, and infection. Multiple studies suggest that platelet interactions with neutrophils, microbial pathogens, and activated endothelium promote biological processes that lead to the consumption and/or receptor-mediated clearance of endogenously produced and transfused platelets. In septicemic patients, it has been hypothesized that bacteria in the bloodstream can trigger platelet destruction and shorten lifespan through the induction of vascular damage, which lead to platelet consumption, while drug-induced thrombocytopenia is usually considered to be immune-mediated and clinically, drug-induced platelet-specific autoimmunity resembles acute immune thrombocytopenic purpura. Although several mechanisms for drug-induced antibody formation have been described, some drugs implicated with platelet antibodies are thought to result when a drug interacts with platelet membrane glycoproteins (Table 16.2). HSCT is associated with an impaired response to platelet transfusion during or after complications such as fever, infection, acute graft-versus-host disease (GvHD), and sepsis, which have been shown as factors affecting platelet transfusion efficacy [12, 13].

16.2.4 Clinical Features

In general, the increase in platelet count after transfusion depends on the number of platelets in the transfused blood components and their distribution within the body. The corrected count increment (CCI) is widely used to determine PTR and can be easily calculated using the following formula:

$$\mathrm{CCI} = \left[\left(\frac{\text{Absolute count increment}}{\mu\mathrm{l}}\right) \times (\text{body surface area, m}^2)\right] \Big/ (\text{Number of platelets transfused} \times 10^{11})$$

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Table 16.2 Drugs reported to cause drug-dependent platelet antibodies.

Drugs
Abciximab
Carbamazepine
Ceftriaxone
Eptifibatide
Heparin
Oxaliplatin
Phenytoin
Piperacillin
Piperacillin/tazobactam
Quinidine
Quinine
Rifampin
Sulfamethoxazole/trimethoprim
Tirofiban
Vancomycin

Studies have shown that platelets need at least 60 minutes to equilibrate in the intravascular space; therefore, platelet counts should be measured 1 hour after the end of the transfusion. Assessing platelet transfusion efficacy is essential, and PTR is defined as an insufficient increase in platelet count at 1 hour or 24 hours after transfusion. Evaluating poor post-transfusion platelet recovery at different times is crucial for distinguishing between alloimmune and non-alloimmune causes. Most studies define PTR as a CCI of <5,000 after two sequential transfusions of ABO-matched and fresh platelets. However, it is generally accepted that a CCI $<7.5 \times 10^9$/l at 1 hour is strongly suggestive of alloimmune refractoriness. A reasonable increase at 1 hour that falls to $<7.5 \times 10^9$/l at 24 hours post-transfusion is, on the contrary, suggestive of non-immune causes. Nonimmune factors, which are thought to account for 80% of cases, lead to increased platelet consumption. Bleeding, infection/sepsis, splenomegaly, and GvHD in patients undergoing allogeneic HSCT are the most common nonimmune causes of refractoriness to platelet transfusions. Immune factors are responsible for PTR in the remaining 20% of cases. As already mentioned, anti-HLA antibodies are the leading factors involved, followed by anti-HPA antibodies and the combination of the two [8, 14, 15].

16.2.5 Diagnosis

A screening test for the presence of anti-HLA or anti-HPA antibodies should be performed when PTR is suspected. Today, solid-phase tests use beads coated with

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individual HLA antigens to identify antibody specificities and their strength (avidity) or amount of antibody binding, expressed as the mean fluorescent intensity (MFI). The increased sensitivity of the solid-phase assay can create problems because the clinical significance of low-level antibodies is not always associated with PTR. The solid-phase assay that targets complement-fixing antibodies could improve the association between anti-HLA antibodies and PTR. However, more studies are needed to calibrate the results of this assay in PTR [16, 17].

16.2.6 Therapy

16.2.6.1 Prevention

The introduction of restrictive platelet transfusion is the first way to reduce the risk of refractoriness in hematological patients if repeated transfusions are required. Stringent leukoreduction performed after the collection of blood components has proven to be effective in reducing the alloimmunization risk. The use of platelet additive solutions also decreases the transfer of soluble HLA antigens from the donor to recipient. However, alloimmunization mechanisms are complex and highly dependent on the recipients' own HLA group, disease, and treatments; as a result, some cases of alloimmunization cannot be prevented [18, 19].

16.2.6.2 Treatment

Finding a compatible platelet unit for an alloimmunized patient depends on the tests available, the frequency of the patient's HLA type relative to the pool of HLA-matched donors, and the level of alloimmunization. The platelet crossmatch is usually the fastest and easiest way to obtain a compatible unit. The crossmatch approach is commonly used because compatible units usually can be obtained within 24 hours, and both HLA and HPA compatibility issues are covered with no additional testing. However, there are not enough platelets available to make compatibility tests for hypersensitized patients, which limits finding a compatible match. To provide compatible platelets for refractory patients, a large inventory of apheresis platelet units would have to be available. It has been demonstrated that a median of 70 donors should be tested to provide crossmatch-compatible transfusion units. The second protocol involves selecting HLA-matched platelets; patients can be supported with apheresis platelets from donors whose HLA-A and HLA-B antigens match those of the patient. However, obtaining a supply of 4/4 matches is possible only for blood centers that have a large number of HLA-typed donors and a well-organized inventory. These strategies require a large HLA-typed donor pool, and even then, there is no guarantee that full matching for patients with broad immunization or rare haplotypes will be achieved. In addition, these approaches are time-consuming and expensive. Blood centers that cannot track the HLA type of units in inventory must resort to recruiting known donors, which causes delays in obtaining units and usually allows a very limited supply of platelet units. In addition, anti-HLA-C antibodies have demonstrated an involvement in PTR, and therefore, HLA-C should also be considered in the classification system

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for matching degrees. Interestingly, a new matching strategy based on HLA epitopes has been proposed. Specific regions of polymorphism in HLA molecules determine the nature of public and private HLA epitopes, which are shared by multiple HLA antigens. Matching at the epitope level, based on either the characterization of short sequences of amino acids from linear or discontinuous regions of the HLA molecules, may be more relevant for assessing HLA compatibility between patients and donors and the effect of donor-specific anti-HLA antibodies. This approach of epitope matching can result in more efficient matching, particularly for highly sensitized patients. HLA Matchmaker software can evaluate HLA compatibility based on HLA epitopes, and HLA epitope-matched platelets have been shown to be not inferior to traditional HLA-matched platelets. Studies have found that the HLA Matchmaker approach successfully identified donors associated with good transfusion outcomes in refractory recipients.

Although the incidence of anti-HPA antibodies causing PTR is low, this possibility should be investigated when most of the crossmatches are incompatible or when the HLA-matched transfusions fail. If antibodies against HPA are present, then donors of known platelet antigen phenotype may be recruited. The patient's relatives, who may share the patient's phenotype, should also be tested.

Although some papers have reported no difference nor superior platelet increments from single-donor units compared with pooled concentrates administered to hematological patients without refractoriness, other reports have described a small but potentially clinically relevant benefit in transfusing pooled random-donor platelets compared with single-donor units for patients with PTR of any etiology. The data appear to support the practice of switching to the use of pooled products when refractoriness may be present in patients who are awaiting matched products and class I HLA antibody test results [20–25].

16.2.7 Future Perspectives

In hematological patients, several measures have been reported to overcome PTR and reduce the presence of alloantibodies, such as intravenous immunoglobulins (IVIGs), plasma exchange, protein A column therapy, or rituximab. In HSCT, PTR has been associated with graft failure, delayed engraftment, early mortality, and decreased overall survival. In nonresponsive cases after the previous therapies, splenectomy has been described as an option to improve the yield of platelet transfusion, although it does not alter the number and type of anti-HLA antibodies.

Some data suggest that complement activation may play a more important role in the pathophysiology of platelet HLA antibody-mediated refractoriness. Fc regions of IgG HLA antibodies are able to bind the first component (C1q) of the classical (antibody-dependent) complement pathway, resulting in complement deposition. Eculizumab, a monoclonal antibody that binds and inhibits C5 complement, has been used to treat PTR in alloimmunized patients with severe thrombocytopenia, potentially allowing enough time to acquire better or fully HLA-matched platelet products or until platelet recovery occurs following chemotherapy or HSCT.

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Thrombopoietin receptor agonists have shown promising effects in treating thrombocytopenia. Few studies have focused on the application of avatrombopag, reported as an effective and safe treatment in PTR.

Several studies report cases of 24-hour continuous infusion of platelets in patients with PTR. This approach might be a feasible and safe strategy when HLA/HPA-compatible platelets are not available, as well as efficacious in treating and preventing hemorrhage in patients with hematological diseases and chemotherapy-induced thrombocytopenia.

Reduction or even elimination of HLA antigens on donor platelet surfaces to reduce the immunogenicity of platelets while preserving their normal physiological function has been a topic of interest for the prophylaxis and treatment of HLA alloimmunization and refractoriness. Although successful cases are rare, the findings indicate the potential feasibility of using HLA-stripped platelets. Nevertheless, it should be noted that the function of platelets after acid treatment may be compromised. In biological and gene-editing technology, HLA-universal platelets, which refer to blood components with reduced or no HLA antigen expression that can be supplied continuously, have been successfully produced in vitro. This is an appealing perspective in the field of transfusion medicine that will not only alleviate the shortage of resources but also prevent HLA alloimmunization and refractoriness.

Other studies provide strong suggestions that storage-induced platelet apoptosis can be a risk factor for alloimmunization after platelet transfusions, and investigations aiming at abolishing or minimizing platelet apoptosis during storage are warranted. Also, the effect of pathogen inactivation on alloimmunization should be investigated; indeed, leukofiltration plus pathogen reduction prevented alloimmune PTR in a dog transfusion model. These methods are effective with apheresis platelets and platelet concentrates and may be particularly important when supporting hematological patients [26–32].

16.3 Transfusion-Related Adverse Events

16.3.1 Febrile Nonhemolytic Transfusion Reactions

16.3.1.1 Definition

A febrile nonhemolytic transfusion reaction (FNHTR) is defined by a temperature increase ($\geq$38 °C or $\geq$1 °C above baseline) during or shortly after transfusion, generally within 4 hours from the end of the procedure, without any other cause explaining the signs or symptoms observed. Thus, FNHTR is a diagnosis of exclusion. Chills, rigors, tachypnea, and other manifestations may accompany fever (see below).

16.3.1.2 Incidence

Among the transfusion-associated adverse events, FNHTR is the most frequent, with a reported range rate of 1 : 800–1 : 1,000 transfusions in the US [33] and around

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1 : 3,900 in 2021 in Italy, accounting for 40.4% of all the adverse events in this country [34].

16.3.1.3 Pathogenesis

Mechanisms include immune and nonimmune etiologies, with the former being caused by anti-HLA antibodies in the recipient, reacting against donor lymphocytes, granulocytes, and platelets. Antibodies against antigens other than HLA specificities may be involved, like HPA or other granulocytic-specific antigens [33, 35]. Nonimmune etiology is recognized by the presence of cytokines in the product during storage and their release into the recipient's circulation during and after transfusion.

16.3.1.4 Clinical Features and Preventive Measures

Clinical manifestations include fever ($\geq$38 °C) possibly associated with chills, headache, and tachypnea occurring during or after transfusion, generally within 0–4 hours. Due to the relatively high frequency and the partial or total transfusion ineffectiveness associated with FNHTR, it represents a substantial burden on patient care and mitigation strategies are in place, such as pre-storage leukoreduction and the use of solvent detergent [36]. Leukoreduction allows for removing the donor antigens that are the targets of recipient antibodies causing immune FNHTR and therefore is the main mitigation strategy, demonstrating to reduce this adverse effect to 50% [37]. The use of solvent detergent compared with plasma contributes to lower the donor-derived cytokines in the product or the donor antibodies against recipient's white blood cells [33]. Previous pregnancies and multiple transfusions are recognized as the main risk factors for FNHTR, due to immunization secondary to transfusion or recall of prior recipient antibodies following transfusion [38].

16.3.2 Transfusion-Related Acute Lung Injury

16.3.2.1 Definition

The development of acute pulmonary edema within 6 hours of transfusion might suggest a transfusion-related acute lung injury (TRALI), whose diagnosis is mainly based on clinical factors including bilateral lung infiltrates, hypoxemia, tachypnea, tachycardia, and hypotension. It is clinically indistinguishable from acute respiratory distress syndrome (ARDS), and attempts to standardize definitions of TRALI have been made around 20 years ago in order to overcome the prior lack of consensus, hindering its clinical recognition and research investigation in the past [39].

16.3.2.2 Incidence

Although less frequent than FNHTR, TRALI is associated with significant morbidity and higher mortality compared with other transfusion-related diseases [40]. Nonetheless, it is important to note that predisposing conditions (surgery, infection, cytokine therapy, chronic alcohol use, tobacco use, and systemic inflammation) contribute to patient outcomes. The National Blood Collection and Utilization Survey (NBCUS) reported a range rate for TRALI of 1 : 57,000 and 1 : 64,000 transfusions in 2011, 2013, and 2015 [33], and only two confirmed

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cases out of over 2,800,000 transfusions have been documented by the Istisan Report in Italy in 2021 [34]. However, underreporting cannot be excluded due to underrecognition (i.e. differential diagnosis with ARDS) or the lack of uniform diagnostic criteria during the past years.

16.3.2.3 Pathogenesis

Both human studies and murine models support a "two-hit" pathogenetic hypothesis, where a predisposing clinical condition (see above) acts by priming neutrophils and activating pulmonary endothelial cells in a recipient of a blood product containing donor-derived anti-HLA and anti-HNA antibodies against the cognate ligands present in the recipient [39, 41, 42]. In 20% of cases, no anti-HLA or anti-HNA antibodies are identified [43], and a small number of TRALI cases are caused by recipient anti-HLA antibodies reacting to HLA antigens present in donor leukocytes contained in the blood product ("reverse" TRALI) [44]. The binding of antibodies to their cognate antigens together with a predisposing clinical condition induces neutrophil activation and subsequent release of inflammatory cytokines and toxic oxygen metabolites, leading to endothelial damage at the alveolar-capillary level and finally to lung injury. Both class I and class II anti-HLA antibodies have been associated with severe manifestations [45].

16.3.2.4 Clinical Features and Preventive Measures

As already mentioned, TRALI occurs within 6 hours after transfusion; signs and symptoms include tachypnea, tachycardia, hypoxia, fever, and bilateral lung infiltrates, mimicking ARDS. Although transfusions with plasma or whole blood are mostly associated with TRALI, all the other blood products might cause this adverse effect [39]. The Laboratory of Histocompatibility and Immunogenetics contributes to the diagnosis of TRALI by identifying the donor-derived anti-HLA or anti-HNA antibodies in the recipient after transfusion and in the respective donor, performing the HLA/HNA typing of patient and eventually a leukocyte crossmatch between the donor serum and patient cells. Mitigation strategies have been adopted by different countries with effective reduction of TRALI incidence [46], mostly thanks to the switch of plasma donation for clinical purpose only from male or nulliparous female donors not having received prior transfusion, or even screening plasma donors for leukocyte antibodies. The UK was the first country to start this process and now TRALI is a rare occurrence, as reported by the UK hemovigilance [47]. Pooling of products is an additional strategy, leading to dilution of any antibodies or their neutralization by residual leukocytes or soluble HLA/HNA molecules contained in the pool [39]; also, the use of solvent detergent plasma proved to be effective in reducing the incidence of this transfusion-associated life-threatening complication [46].

16.3.2.5 Future Perspectives

Despite advances in the understanding of pathogenic mechanisms, current treatment is only supportive care and TRALI remains a significant clinical problem. Nonetheless, innovative therapies have been recently reviewed as IL-10 treatment, downregulation of C-reactive protein levels, targeting reactive oxygen species, and blocking IL-8 receptors have shown to be promising approaches [48].

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16.3.3 Transfusion-Associated Graft-Versus-Host Disease

16.3.3.1 Incidence

Donor lymphocytes contained in the blood product might, in some circumstances, engraft and expand in the recipient, causing a transfusion-associated graft-versus-host disease (TA-GvHD). A very rare condition, TA-GvHD, is often lethal with a 90–100% mortality rate [49, 50]. Reported rates range between 1 : 950,000 and 1 : 13,000,000 [33], and half of the cases occur in patients not predicted to be at risk of TA-GvHD and thus who did not receive irradiated blood components [51].

16.3.3.2 Pathogenesis

The HLA system is involved in that donor antigens are shared by the recipient, who therefore do not recognize the donor as non-self [52, 53]: this allows the donor lymphocytes contained in the blood product to evade the HLA-mediated immune detection by the recipient and expand to react against the recipient tissues.

16.3.3.3 Clinical Features

TA-GvHD mostly occurs within 30 days after transfusion, with a median of 11 days (interquartile range: 8–14 days); clinical manifestations include skin rash (80% of cases), followed by fever (67.5%), liver enzyme elevation (66.4%), pancytopenia (65.2%), diarrhea (43.1%), bone marrow aplasia (22.7%), and hepatomegaly (13.5%) [51].

16.3.3.4 Diagnosis

Diagnosis is based on the demonstration of donor-derived cells, chromosomes, or DNA in the recipient affected tissues or blood. This can be obtained by chimerism detection [54], strengthened by HLA typing of donor and recipient who is expected to show some degree of sharing. Chimerism is tested on blood or on the affected tissue(s) by biopsy. A recent systematic review found that donor HLA antigens were not foreign to the recipient in more than two-thirds of cases [51].

16.3.3.5 Preventive Measures

Although the relative contribution of recipient and blood component factors is not fully understood, irradiation is universally recognized as the main method of inactivating lymphocytes in the transfused component and thus represents the major technology to prevent TA-GvHD [55]. Immunosuppression of the recipient and HLA sharing between the recipient and donor mandate the irradiation of the blood product before transfusion. There are guidelines providing more details on the conditions and the diseases triggering irradiation [55–57]. Interestingly, Kopolovic and colleagues [51] found that there were no cases implicating blood components stored for more than two weeks, raising the hypothesis that storage is associated with lymphocyte inactivation. This finding is also consistently found in other studies [50, 58]. Of note, leukoreduction is not sufficient to prevent TA-GvHD alone. The most involved components are red cells and whole blood, although platelets and

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buffy coats were identified as the responsible components in some published cases [51].

16.3.4 Post-Transfusion Purpura

16.3.4.1 Definition

Severe thrombocytopenia occurring 2–14 days after a platelet-containing blood product should prompt investigations on a potential post-transfusion purpura (PTP), a rare adverse event that might be even underrecognized due to the apparent similarity with other thrombocytopenic conditions.

16.3.4.2 Incidence

The incidence ranges between 1 : 24,000 and 1 : 100,000 transfusions [33, 59], and the first case was described in 1959 in a female patient who developed severe thrombocytopenia after a single blood transfusion [60].

16.3.4.3 Pathogenesis

Typically, patients are female multiparous recipients with anti-HPA antibodies against the cognate antigen in the blood component(s); sensitization of the recipient mostly occurs through prior pregnancy or by receiving transfusion products containing the same platelet antigen(s) that sustain PTP, by a mechanism of immunological recall leading to an anamnestic antibody response. The mechanism leading to the destruction of autologous platelets is less defined, and current hypotheses involve the potential role of soluble HPAs or the concomitant production of platelet autoantibodies [61, 62]. Anti-HLA antibodies have been proposed as a potential cause [63], but, conversely to the setting of PTR, testing is not useful here.

16.3.4.4 Clinical Features

Thrombocytopenia is generally severe (platelets $<10 \times 10^9$/l), and mucocutaneous, gastrointestinal, or urinary tract bleeding are the main clinical manifestations, with a fatality rate of 10–20% mainly attributable to intracranial hemorrhage [59, 64]. Fever, chills, and PTR might be accompanying manifestations [65].

16.3.4.5 Diagnosis

The Laboratory of Histocompatibility and Immunogenetics supports the diagnostic workup by performing antiplatelet antibody testing and the HPA genotyping. Both direct and indirect testing can be used to find the platelet-specific antibodies in the recipient; those antibodies are directed against the HPAs expressed on the platelet glycoproteins. Antibodies directed against HPA-1b, either alone or in conjunction with other anti-HPA antibodies, are the most reported followed by anti-HPA-1a and anti-HPA-5b. Antibodies detected against other specificities include HPA-2a, HPA-2b, HPA-3a, HPA-3b, HPA-4a, HPA-5a, HPA-15b, and GPIV/CD36. Besides this, the HPA genotyping of the recipient is useful to confirm the antibody specificity as well as to guide future transfusion strategies in sensitized patients, such as the specific selection of blood products from known genotyped donors [59].

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16.3.4.6 Preventive Measures and Future Perspectives

All the platelet-containing blood products are involved as whole blood, red cells, platelets, and plasma have been reported to have induced PTP [66]. Overall, PTP cases have dropped after the introduction of leukoreduction [67], although a definite explanation is lacking; studies show that leukoreduction of whole blood allows for a 100-fold reduction of platelet contamination [68]. Of note, the reappraisal of whole blood use for trauma is expected to expose patients to more platelet antigens compared with separated blood components, thus potentially increasing the risk of PTPs in the near future [59].

16.4 Neonatal Alloimmune Neutropenia

16.4.1 Definition

Neonatal neutropenia in the White population is generally defined when absolute neutrophil counts (ANCs) are $<1 \times 10^9$/l, even though in the first week of life, the lower limit of ANC is 2.5×10^9/l [69, 70]. Neutropenia is common in neonatal ICUs, especially in pre-terms, and is mostly due to nonimmune disorders, such as maternal hypertension/pre-eclampsia, neonatal infections, twin–twin transfusions, and maternal drugs [71, 72].

When neonatal neutropenia is due to maternal alloantibodies, the condition, first described over 60 years ago by Lalezari et al. [73], is known as neonatal alloimmune neutropenia (NAIN; also referred to as NAN or NIN) and shares similar pathogenesis with FNAIT and with hemolytic disease of the fetus and newborn (HDFN) [71].

16.4.2 Incidence

The incidence of NAIN is not easy to establish for several reasons: many cases are undetected because they show mild or no symptoms, clinicians do not routinely ask for laboratory testing for their patients, and the laboratory tests are complex and require experienced laboratories [71, 72]. A study by Zupańska et al. on over 24,000 newborns indicates that the incidence is approximately 1 per 6,000 neonates [74].

16.4.3 Pathogenesis

HNA antigens are epitopes expressed on glycoproteins present on the membranes of neutrophils; 14 HNAs subdivided into five HNA systems have been described so far [75]. NAIN occurs when the mother is sensitized to fetal HNAs inherited by the father and foreign to her. Anti-HNA alloantibodies of the IgG class cross the placenta and bind to fetal neutrophils, destroying them [71]. It is unclear whether anti-HNA antibodies also have an inhibitory effect on granulocytopoiesis [72]. NAIN can occur in first pregnancies, indicating that alloimmunization may take place during pregnancy (or even before the first pregnancy). The most frequently detected alloantibodies in NAIN are specific for HNA-1a, HNA-1b, and HNA-2 [71, 72].

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Table 16.3 Clinical data from two case series.

First author, year of publication [reference]	No. of confirmed NAIN cases	ANC at onset	Three most frequent HNA-antibody specificities (%)	Neonates with clinical symptoms %	Treatment	Outcome
Cattaneo 2023 [77]	31	Median: 330/ml (range: 40–1,090)	Anti-HNA-1b: 42% Anti-HNA-1a: 33% Anti-FcgRIIIb: 13%	19.4% Severe infections requiring hospitalization	6/31 symptomatic neonates (19.4%) received G-CSF 2/31 received prophylactic G-CSF 2/31 received intravenous immunoglobulin	Median time to recovery 31 days (range: 8–180). One pre-term neonate required 180 days
van den Tooren-de Groot 2014 [79]	35	Mean: 430/ml (range 0–1,200)	Anti-HNA-1b: 34.2% Anti-HNA-2 : 22.9% Anti-HNA-1a: 20%	60% Urinary tract infection, omphalitis, candida mucositis, FUO, sepsis	25/35 neonates (71%) were treated with antibiotics 1/35 received G-CSF	0% mortality

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16.4.4 Clinical Features

Most probably, the majority of NAIN cases are undetected because of a lack/absence of clinical symptoms. Newborns diagnosed with NAIN usually have very low ANC ($<0.5 \times 10^9$/l), and the main symptoms are omphalitis, fever of unknown origin, skin infections, meningitis, and pneumonia [76]. Though the majority of infections are mild, severe life-threatening sepsis can occur [71]. Since neutropenia is caused by maternal alloantibodies, the disorder is self-limiting [71]; the duration of neutropenia ranges from 7 to 11 weeks [77, 78]. Table 16.3 summarizes clinical and laboratory data from the papers by Cattaneo et al. [77] and van den Tooren-de Groot et al. [79], which report two series of NAIN cases.

16.4.5 Diagnosis

Differential diagnosis is with the other types of immune neutropenias, namely neonatal autoimmune neutropenia and autoimmune neutropenia of infancy [70, 71, 76]. The investigations for NAIN requested to the Laboratory of Histocompatibility and Immunogenetics include the detection and identification of maternal HNA alloantibodies and HNA genotyping of the mother, father, and infant. Though not mandatory, a crossmatch with maternal serum and paternal neutrophils is useful [72, 75].

16.4.6 Therapy

Treatment of symptomatic NAIN is based on antibiotics, which are usually sufficient in managing infections in these neonates. Due to the often mild and self-limiting course of NAIN, antibiotic prophylaxis is not recommended [72, 80]. Granulocyte-colony-stimulating factor (G-CSF) has been shown to increase ANC in NAIN cases [72], but there is controversy about its therapeutic effectiveness [81]. In cases that are refractory to G-CSF, IVIGs have been successfully used as second-line treatment [82]. Since in some cases NAIN can have a severe course, neonatologists should be encouraged to reach a timely diagnosis in order to prevent serious infections [72, 77]. As regards laboratory testing, it is expected that in future, more reliable HNA antibody techniques will become available, improving the diagnostic process of NAIN [75].

16.5 Fetal and Neonatal Alloimmune Thrombocytopenia

16.5.1 Definition

FNAIT is a rare condition resulting from HPA incompatibility between a mother and her fetus. This incompatibility prompts the mother's immune system to produce antibodies that cross the placenta and target the fetus's platelets, leading to their destruction. Although platelet function remains intact, the most severe complication is intracranial hemorrhage.

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16.5.2 Incidence

The incidence of FNAIT is estimated to be between 1 in 1,000 and 1 in 10,000 births [83–85]. In 2014, Kamphuis et al. published a review reporting that 0.15% of neonates were born with severe thrombocytopenia (platelet count $<50 \times 10^9$/l), and FNAIT was diagnosed in 27% of these cases [86]. Additionally, Kamphuis et al.'s data indicated that 25% of the neonates with FNAIT experienced intracranial hemorrhage.

16.5.3 Pathogenesis

FNAIT occurs when fetal platelets express an HPA inherited from the father but absent in the mother. This leads to alloimmunization in the mother, causing her to produce IgG antibodies against the fetal HPA. These antibodies cross the placenta, bind to the fetal platelets, and remove them from circulation, resulting in fetal or neonatal thrombocytopenia. In some cases, the maternal anti-HPA antibodies can also suppress megakaryopoiesis, further worsening the thrombocytopenia [87].

The HPA-1a antigen is implicated in 85% of FNAIT cases. Typically, the mother is homozygous for HPA-1b, while the father is either homozygous or heterozygous for HPA-1a. About 98% of the White population is either HPA-1a/1a or HPA-1a/1b. Anti-HPA-1a IgG antibodies are detected in approximately 10% of HPA-1b/1b women [88, 89]. The second most common antibody causing FNAIT in Whites is anti-HPA-5b [90], whereas in Asians, FNAIT is associated with the HPA-4 system [91, 92]. Alloimmunization to HPA is strongly associated with the HLA-DRB3*01:01 allele [89, 93].

16.5.4 Clinical Features

Clinical signs of FNAIT range from asymptomatic mild thrombocytopenia to severe thrombocytopenia, which can cause spontaneous intracranial bleeding, leading to severe neurological damage or death [84, 94, 95]. In most cases, diagnosis occurs at the birth of an affected neonate. Occasionally, HPA incompatibility between parents is identified before birth through antenatal screening or when testing is conducted because the mother's sister had an affected neonate.

FNAIT can occur during a first pregnancy, as demonstrated by the "No IntraCranial Haemorrhage (NOICH) registry," which shows that 63% of FNAIT cases were diagnosed in first pregnancies [96].

The clinical condition of neonates diagnosed with FNAIT correlates with the severity of thrombocytopenia. Neonates with mild (platelet count between 50 and 99×10^9/l) or severe thrombocytopenia ($<50 \times 10^9$/l) may exhibit petechiae, ecchymosis, and bleeding. In a series of 123 FNAIT neonates [97], the distribution of clinical signs was as follows: no symptoms (34%), only cutaneous bleeding (47%), intracranial hemorrhage (14%), and bleeding in other sites (2%).

16.5.5 Diagnosis

A diagnosis of FNAIT should be considered in any apparently healthy term neonate presenting with unexplained thrombocytopenia within 24–48 hours after birth or in

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cases of intracranial hemorrhage. Laboratory tests for diagnosing FNAIT include anti-HPA screening and identification in maternal sera, as well as HPA typing of both the mother and father. If a maternal anti-HPA alloantibody is detected against a paternal antigen, FNAIT can be confirmed. Additionally, fetal HPA genotyping is now available and can be useful for managing FNAIT cases [98].

16.5.6 Therapy

For neonates diagnosed with FNAIT who require an urgent platelet transfusion, random platelets can initially be used to raise the platelet count [99, 100]. However, these transfused platelets will not last long due to the maternal anti-HPA antibodies, and thus obtaining compatible units is crucial. The preferred option is to collect platelets from the mother, which should be washed and irradiated. Alternatively, platelets from HPA-typed compatible donors can be used [97].

It is common practice to treat FNAIT neonates with IVIG, although the evidence supporting its efficacy is not very strong [100].

In families with an affected fetus or neonate, the risk of recurrence in future pregnancies depends on whether the father is homozygous (100% risk) or heterozygous (50% risk) for the relevant HPA. In the latter case, fetal genotyping can assist in clinical decision-making [98].

16.6 Conclusions

Transfusion medicine is a rapidly evolving field, and some of the elements presented in the current chapter might change in the future, especially with respect to the incidence and to the mitigation strategies, with the latter evolving together with scientific and technological advances and responding somehow to logistical and economic challenges. Despite being rare, the diseases in the field of transfusion medicine are clinically relevant and some of them are associated with elevated morbidity and mortality. Hemovigilance plays a fundamental role in detecting and monitoring the incidence of transfusion-related adverse reactions, their outcomes, and the effects of the preventive measures adopted. Moreover, it offers a dynamic landscape and finally contributes to the improvement of the field. Specific knowledge of the HLA, HPA, and HNA systems in this context appears of utmost importance for the optimal management of such situations.

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Part III

Methods in Immunogentics and Histocompatibility

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17

Isolation and Purification of Test Material

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17.1 DNA Extraction

DNA extraction is a fundamental process in molecular biology. DNA can be isolated from any biological material that has been isolated from a sample, typically a biological sample such as blood, saliva, or tissue, for subsequent testing, analytical or preparative purposes, for example, for polymerase chain reaction (PCR) sequencing, cloning in molecular biology, or for forensic purposes. Historically, the manual process of extracting and purifying nucleic acids has been complicated, time-consuming, and labor-intensive and is limited to low throughput [1]. Since the first DNA isolation was successfully done by Friedrich Miescher in 1869 and the initial DNA extraction developed from density-gradient centrifugation strategies by Meselson and Stahl in 1958, many techniques for biomolecule purification have been developed.

The process involves three main steps: lysis, precipitation, purification and elution.

1) **Lysis**: In lysis, the cells are broken down to release the DNA from the nucleus, which could be a mechanical, chemical, or enzymatic procedure. Lysis buffer usually contains a detergent (e.g. SDS) to break down cell membranes and a buffer (e.g. Tris-HCL) to maintain pH. Proteinase K, an enzyme that digests proteins, including nucleases that could degrade the DNA, may be added to the buffer. Optionally, RNase A could be added to the sample to degrade RNA. This will ensure a pure DNA sample.
2) **Precipitation**: The second step is called precipitation. To obtain pure DNA, the DNA is separated from proteins and other cell components by precipitating the DNA with ethanol or isopropanol. DNA is insoluble in these alcohols and will aggregate together as a pellet, which can be taken out by a hook or simply by centrifugation. The precipitation can be improved by increasing ionic strength, normally by adding sodium acetate.
3) **Purification and elution**: The next step is purification. At this stage, the DNA is precipitated and can be eluted and stored using Aqua bidest or low-concentration TE (Tris-EDTA) buffer.

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There are currently a number of methods that can be used to extract DNA, such as solution-based and column-based protocols.

The manual methods available from various companies are complete kits containing most of the components needed for nucleic acid isolation. However, most of them require repeated centrifugation steps, followed by the removal of the supernatant and additional mechanical treatment, depending on the type of sample. In recent years, there has been increasing demand for automated systems for medium-to-large laboratories [2].

Organic extraction: This involves incubation in several different chemical solutions, including a lysis step, phenol–chloroform extraction, ethanol precipitation, and washing steps. Because it is inexpensive and yields large amounts of pure DNA, organic extraction was often used in laboratories. However, it has the disadvantage of using the toxic chemicals phenol and chloroform, and there is an increased risk of contamination due to the transfer of DNA between multiple tubes.

The Chelex extraction method: This involves adding Chelex resin to the sample. The solution is boiled, vortexed, and centrifuged. The Chelex method is much faster and simpler than organic extraction, and only one tube is needed, reducing the risk of DNA contamination. Unfortunately, Chelex extraction does not yield as much and the DNA is single-stranded, meaning it can only be used for PCR-based analysis.

Solid-phase extraction: This method takes advantage of the fact that DNA binds to silica, for example, using a spin column-based extraction method. A column containing silica gel or silica beads and chaotropic salts is applied to the sample containing DNA. By making the nucleic acids hydrophobic, the chaotropic salts disrupt the hydrogen bonds between the strands and facilitate the binding of the DNA to the silica. The DNA binds to the silica, while the rest of the solution is washed with ethanol to remove chaotropic salts and other unwanted components. The DNA can then be rehydrated with aqueous low-salt solutions, allowing the DNA to be eluted from the beads.

High-quality, largely double-stranded DNA is obtained using this method. It can be automated and has a high throughput. It is a one-step method, that is, the entire procedure is completed in one tube, which reduces the risk of contamination. Several commercial solid-phase extraction kits are produced and marketed by different companies.

Beads method: Magnetic beads have emerged as an efficient and reliable tool in the automation of nucleic acid extraction workflows. Extraction based on magnetic beads is based on the use of paramagnetic particles with a coating that is capable of binding specifically to nucleic acids. These beads only exhibit magnetic properties when an external magnetic field is applied, which allows them to be separated easily and without clumping. There are two different versions of bead-based DNA isolation on the market. First, the whole reaction is performed directly in a pipette tip or the magnetic device is coated with a plastic sleeve. The beads covered with DNA are transferred from one well to the other by means of the tools. Using

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magnetic beads for nucleic acid extraction enables automated high-throughput extraction workflows, producing high yields of high-quality DNA and RNA.

There are four main stages:

1) **Lyse**: To release nucleic acids, cell membranes and nuclear envelopes must first be disrupted. The lysis of the samples is carried out in buffer systems that are specifically designed for each type of starting material.
2) **Binding**: After lysis, in the presence of a suitable binding buffer, particles consisting of a magnetic core and a nucleic acid-binding coating are introduced into the sample. This selectively binds the targeted nucleic acid to the bead for extraction. A magnetic field is applied using a magnetic separation device. The particles are attracted and separated from the lysate.
3) **Wash**: A series of wash steps removes unbound contaminants, such as proteins, lipids, and other cellular debris. For further processing, this ensures that only the purified nucleic acids bound to the magnetic particles remain.
4) **Elute**: Nucleic acids are eluted from magnetic beads using low-salt buffer or water.

The advantages of nucleic acid extraction based on magnetic beads are the high specificity, the reproducibility, and the possibility to process several samples at the same time. The magnetic separation step simplifies the process and eliminates cumbersome centrifugation steps, making it suitable for automated and high-throughput applications. This method is widely used in molecular biology research, clinical diagnostics, and other fields where the extraction of high-quality nucleic acids is essential for downstream applications such as PCR sequencing or the analysis of gene expression. Automated DNA extraction is shown in Figure 17.1.

17.1.1 DNA Sources

- **Peripheral blood**: Venous blood is drawn into a tube containing EDTA or citrate to prevent it from clotting. This is the primary source for DNA extraction.
- **Tissue**: In special cases, such as the typing of a deceased donor, material from the spleen can be used for DNA extraction. This involves processing the tissue, either mechanically or enzymatically. The DNA is then extracted using a special kit. The use of spleen material results in a very high concentration of DNA.
- **Saliva**: DNA extraction from saliva can provide high-quality DNA with little or no degradation/fragmentation, suitable for a variety of DNA assays, without the expense of a phlebotomist, and can even be purchased by mail order. Over time, saliva is increasingly being replaced by swab DNA, which is easier to handle [3].
- **Swab**: Today, many human leukocyte antigen (HLA) typing tests for unrelated bone marrow donors are performed using a human buccal swab. This is a noninvasive technique and is suitable for testing healthy individuals. In addition, this technique is also used in cytopenic patients and in leukemia patients in blast crises in order to have a way of extracting DNA.

The samples must be processed as soon as they are collected or stored at 4 °C for up to 2 weeks. Unprocessed samples should not be stored at room temperature. Buccal

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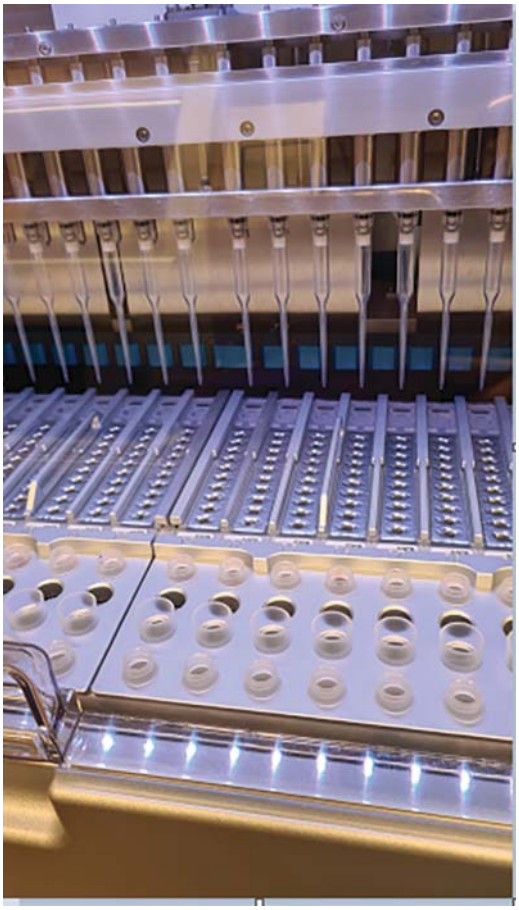

Figure 17.1 Automated DNA extractor, in which 24 samples can be processed simultaneously. The front row contains the tubes for the extracted DNA, the second row contains the pipette tips and tip holders, the third row is empty, and the fourth row contains the blood tubes. Behind them are the cartridges containing the magnetic beads and all the necessary solutions. The blue bar shows the magnet needed for the separation.

swabs contain bacteria and nucleases that degrade DNA. The DNA yield varies and depends on several factors, including the technique used by the person taking the swab, whether the donor is a high or low shedder, and the type of swab used [4].

17.1.2 DNA Storage

Storing DNA is an important aspect of DNA extraction projects as it ensures the integrity and stability of the extracted DNA for downstream applications. For storage at 4 °C, DNA should be resuspended in a special buffer, such as Tris-EDTA (TE). For longer storage, DNA should be kept at −20 °C or better −80 °C. This method preserves the integrity of the DNA. It also slows down the activity of any enzymes that might degrade it.

17.1.3 Quality Control and DNA Measurement

Several quality control techniques can be used to ensure the quality of extracted DNA.

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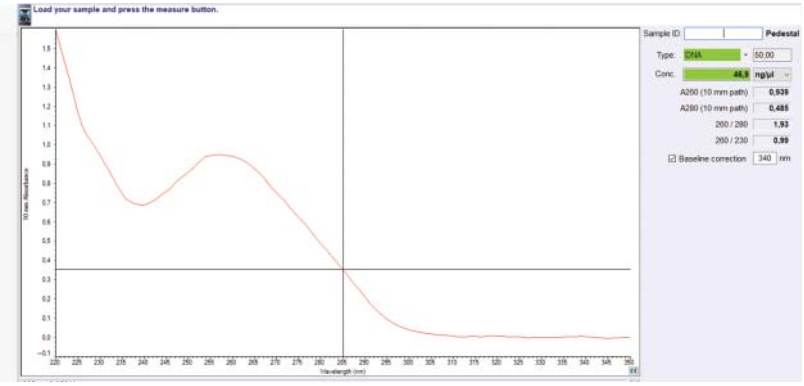

Figure 17.2 DNA measurement with Nanodrop. The photo shows the concentration and the important ratio 260/280.

- **Agarose gel electrophoresis**: An aliquot of the DNA is run on an agarose gel to check the integrity and size of the DNA. This method allows us to check the quality of the DNA directly; it is simple and inexpensive but is time-consuming.
- **Spectrophotometry (e.g. Nanodrop)**: This is a widely used method for the measurement of the concentration and the purity of a sample of DNA (see Figure 17.2). Spectrophotometry measures a sample's absorbance at different wavelengths, typically 260 and 280 nm. The ratio of absorbance at 260 and 280 nm is a measure of the purity of the DNA sample. The ratio should be around 1.8, which is generally accepted as pure for DNA. If the ratio is significantly lower, it may indicate the presence of protein, phenols, or other impurities that absorb strongly at or near 280 nm. The yield of DNA can vary depending on the sample type and extraction method.
- **Fluorometer (e.g. Qubit)**: This instrument uses fluorescent dyes to measure the concentration of DNA and RNA in a sample. It is a rapid and sensitive method that can also be used for the determination of the concentration of DNA samples [5].

17.2 RNA Extraction

RNA is used for qPCR to measure gene expression or for high-throughput RNA sequencing to study the transcriptome. It is also used for reverse transcription into cDNA followed by various downstream applications. RNA extraction is a critical process in molecular biology used to isolate RNA from biological samples, such as cells, tissues, or fluids. RNA is more fragile and prone to degradation than DNA, so the extraction process must be handled carefully to preserve the integrity of the RNA.

Some important points for successful RNA extraction are listed below:

- **Avoid RNase contamination**: RNases are enzymes that degrade RNA. Use RNase-free tips, tubes, and water, and wear gloves to prevent contamination.

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- **Work quickly**: RNA is more susceptible to degradation than DNA, so handle samples promptly and keep them on ice whenever possible.
- **RNase inhibitors**: If necessary, use RNase inhibitors during the extraction process to protect the RNA.

The RNA can be isolated manually using phenol, chloroform, and isoamyl alcohol. Two phases are formed: an aqueous and an organic phase. The RNA is collected in the aqueous phase and can be extracted with isopropanol or ethanol. A method, developed by Piotr Chomczynski and Nicoletta Sacchi, uses a special reagent (trade names and variants include Trizol, TRI Reagent, Trisure, TriFast, STAT-60, RNAzol, or DNAzol), which makes it possible to lyse cells and simultaneously extract RNA and DNA from cells or tissues. This process is based on the so-called single-step method according to Chomczynski and Sacchi. In addition to phenol and chloroform, Trizol contains guanidinium thiocyanate, which lyses the cells and simultaneously inactivates RNases and other enzymes. As a further option for RNA isolation, many kit systems for solid-phase extraction are available from various companies and in numerous designs. These kit systems use small columns or magnetic beads that bind RNA specifically [6, 7].

17.3 cfDNA Extraction

Circulating/cell-free DNA (cfDNA) is predominantly present in the form of nucleosomes, which are nuclear complexes comprising histones and DNA. Additionally, cfDNA has been observed in shorter size ranges and has been linked to regulatory elements [8].

The discovery of cfDNA was first made by Mandel and Metais in 1948. Subsequent studies have demonstrated that the concentration of cfDNA in the plasma of diseased patients is significantly elevated in comparison to healthy individuals. The initial observation of this phenomenon was made in patients with lupus, and subsequent research has revealed that elevated levels of cfDNA are present in over half of cancer patients [9].

cfDNA extraction is a specialized procedure for isolating small fragments of DNA that circulate freely in the blood, plasma, or other body fluids. cfDNA is often derived from apoptotic cells and can be used for noninvasive prenatal testing (NIPT), cancer diagnostics (liquid biopsy), and other clinical applications. The extraction process is more delicate than typical DNA extraction due to the low concentration and fragmented nature of cfDNA. cfDNA can be extracted with different methods (e.g. magnetic beads, silica columns).

The blood collection has to be done in a specialized cfDNA tube containing EDTA (e.g. Streck tubes) or in a tube containing EDTA and a gel membrane. After blood has been drawn, the tubes must be centrifuged within 1 hour to avoid cell lysis of own cells. Streck tubes can be stored uncentrifuged for a few days without any loss of quality.

After centrifugation, the tubes with gel can be stored at −20 °C until the plasma is transferred into an extra tube. The transfer should be performed

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carefully without disturbing the buffy coat or touching the gel layer. A second centrifugation is performed immediately before the cfDNA extraction procedure at 19.000× *g* for 45 minutes at 4 °C to remove any remaining debris. cfDNA extraction can be performed using kits from different companies with similar results.

17.3.1 Quantification and Quality Control

For measuring the concentration of cfDNA, a sensitive method like a fluorometer (e.g. Qubit) or a high-sensitive spectrophotometer should be used.

Quality control can be done by a bioanalyzer to check the fragment size distribution, which shows a peak around 150–200 bp.

Storage of cfDNA should be performed at −20 °C or −80 °C, especially if you want to perform sensitive applications like sequencing or qPCR.

17.3.2 Applications of cfDNA

- **NIPT**: Detecting fetal chromosomal abnormalities or determining the Rhesus factor of the fetus in the case of a Rhesus factor-negative mother, by analyzing cfDNA from maternal plasma;
- **Liquid biopsy for cancer**: Identifying mutations, copy number variations, and methylation patterns in cfDNA from cancer patients;
- **Transplantation monitoring**: Assessing graft rejection by detecting donor-derived cfDNA (dd-cfDNA) in the recipient's plasma. dd-cfDNA values spike initially after a transplant procedure (>5%), with values heavily depending on the transplanted organ and typically drop (<0.5%) within one week for most organs. If the host body rejects the grafted organ, the dd-cfDNA concentration in the blood (plasma) will rise to a level greater than fivefold than those without complications. This increase in dd-cfDNA can be detected prior to any other clinical or biochemical signs of complication. Besides dd-cfDNA in plasma, some research also focused on the excretion of dd-cfDNA through urine. This is of special interest in kidney allograft transplantation [10];
- **Infectious disease monitoring**: Detecting cfDNA from pathogens in blood.

Considerations

- **Low abundance**: cfDNA is present in very low concentrations, so careful handling and efficient extraction methods are crucial.
- **Fragmentation**: cfDNA is typically fragmented, requiring sensitive methods for quantification and analysis.
- **Avoiding contamination**: Contamination by genomic DNA from lysed cells should be minimized, which can occur if blood is not processed promptly.
- This method is highly sensitive and is tailored to preserve and purify small cfDNA fragments, making it suitable for advanced diagnostic and research applications.

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17.4 Cell Preparation

Cell isolation from peripheral blood is an important process in HLA testing (typing, antibody testing, and cross-matching), research, clinical diagnostics, and therapy, allowing the extraction of specific cell types for various analyses or treatments. Besides, peripheral blood spleen and lymph nodes can also be used as sources for cell isolation. An overview of the common steps involved in isolating cells from peripheral blood is listed:

Peripheral blood is collected into tubes with anticoagulants using EDTA, heparin, or citrate to inhibit the clotting of cells. The spleen or lymph node is cut into small pieces, and the cells are washed with phosphate-buffered saline (PBS) and used the same way as diluted peripheral blood.

17.4.1 Density-Gradient Centrifugation

The most used technique is density-gradient centrifugation. The cells, mononuclear cells (e.g. lymphocytes and monocytes), are separated according to their density.

17.4.1.1 Procedure

1) **Dilution**: Blood should be diluted with a buffer, for example, PBS.
2) **Layering**: The diluted blood is layered over a density-gradient medium (e.g. Ficoll–Paque) without mixing the solutions; a different way is to apply the blood with thin glass pipettors (Pasteur pipettes) underneath the Ficoll–Paque (see Figure 17.3).
3) **Centrifugation**: The sample is centrifuged at 400–800× *g* for 20–30 minutes without break. Without break is important for not destroying the buffy coat.
4) **Layer isolation**: After centrifugation, different layers are formed:
   - Plasma (the top layer);
   - Buffy coat (the middle layer containing mononuclear cells);
   - Erythrocytes (the bottom layer) (see Figures 17.4 and 17.5).
5) **Collection**: The buffy coat layer is carefully collected into a new tube for further processing.

Red blood cell (RBC) lysis (if necessary)

If the buffy coat contains residual RBCs, a lysis step could be performed.

A hypotonic lysis buffer (e.g. ammonium chloride solution) or only Aqua bidest should be added. The buffer must be incubated at room temperature until the solution becomes clear, and then the tube must be centrifuged to pellet the white cells and remove the supernatant.

17.4.2 Magnetic-Activated Cell Sorting (MACS)

The cells are labeled with magnetic beads conjugated to antibodies specific to cell surface markers. The cell mixture is passed through a magnetic column, where labeled cells are retained and then eluted.

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Figure 17.3 Separation of leukocytes with Ficoll–Paque solution. Diluted cells are placed in 10 ml glass tubes. Ficoll–Paque is added with a glass Pasteur pipette underneath the blood and the tubes are centrifuged.

17.4.3 Fluorescence-Activated Cell Sorting (FACS)

Cells are stained with fluorescently labeled antibodies targeting specific surface markers. A flow cytometer sorts cells based on their fluorescence characteristics.

17.4.4 Adherence

Certain cell types, like monocytes, adhere to plastic surfaces. Nonadherent cells can be washed away.

These procedures are sufficiently flexible to be adapted to suit the particular requirements of the intended downstream applications.

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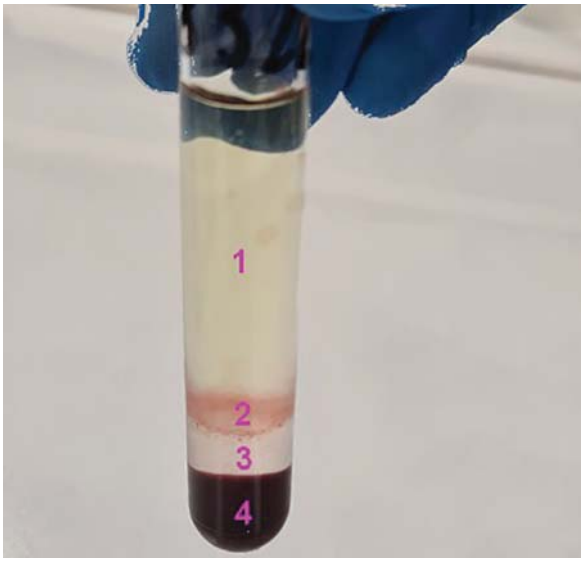

Figure 17.4 Cell separation from venous blood using Ficoll–Paque medium. (1) PBS-diluted plasma, (2) buffy coat with leukocytes, (3) Ficoll–Paque medium, and (4) erythrocytes.

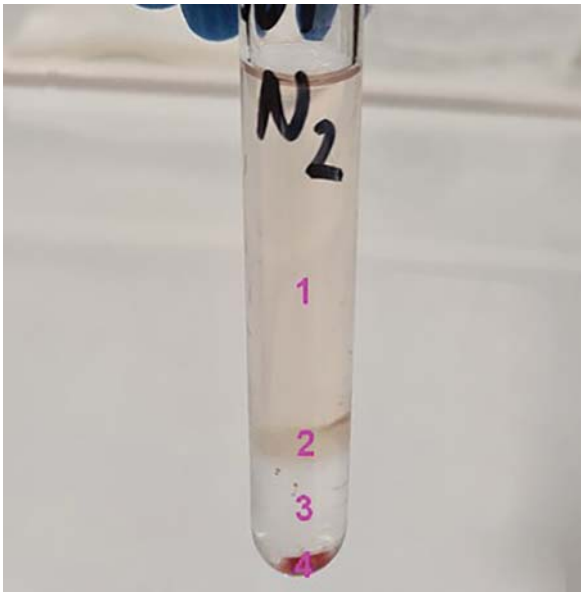

Figure 17.5 Cell separation from spleen using Ficoll–Paque medium. (1) PBS-diluted plasma, (2) buffy coat with leukocytes, (3) Ficoll–Paque medium, and (4) erythrocytes.

17.4.5 Cell Counting and Viability Assessment

After isolation, the viability (with viability dyes, e.g. trypan blue or propidium iodide) and the cell counting are performed. The quality of the cell preparation is frequently contingent upon how the sample is handled, the temperature, the speed of the centrifuge, and the speed of the pipetting [11].

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17.4.6 T and B Cell Separation

T and B cells are separated from peripheral blood. Specific lymphocyte populations are identified based on their unique surface markers.

Separation can be achieved by

17.4.7 Magnetic-Activated Cell Sorting (MACS)

By using CD3 antibodies specific to T-cells and CD19 or CD20 antibodies specific to B-cells are separated via magnetic beads. Cells attached to the magnetic beads will be retained in a column.

- **Positive selection**: The desired T- or B-cells are retained and eluted from the column.
- **Negative selection**: This is an alternative method where non-target cells are labeled and retained, and the target cells (T- or B-cells) are allowed to pass through.

17.4.8 Fluorescence-Activated Cell Sorting (FACS)

FACS sorts cells based on fluorescence intensity after labeling them with fluorescent antibodies specific to surface markers. By using CD3 antibodies specific to T-cells and CD19 or CD20 antibodies specific to B-cells, cells are separated via FACS sort. The stained cells are passed through a flow cytometer, where they are sorted one by one based on the fluorescence detected.

The cytometer separates cells and places them into different tubes or containers based on their fluorescence, resulting in pure populations of T or B cells.

This method is highly precise and can simultaneously separate multiple cell types or subpopulations. However, it requires equipment that is more sophisticated and is more time-consuming than MACS.

17.4.9 Antibody-Dependent Separation

The antibody-dependent separation enriches cells from whole blood by negative selection for the separation of T- and B- cells. Unwanted cells are targeted for removal using tetrameric antibody complexes that recognize CD16, CD19, CD20, CD36, CD56, and glycophorin A in RBCs. When centrifuged over a density medium, the unwanted cells pellet together with the RBCs. The purified lymphoid cells are present as a highly enriched population at the interface between the plasma and the density medium. The desired untouched cells are immediately available for all downstream applications. A second way is to separate the antibody-targeted cells by magnetic beads [12].

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Abbreviations

cfDNA	circulating/cell-free DNA
dd-cfDNA	donor-derived cell-free DNA
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescence-activated cell sorting
HLA	human leukocyte antigen
MACS	magnetic activated cell sorting
NIPT	noninvasive perinatal testing
PCR	polymerase chain reaction
PBS	phosphate-buffered saline
RBC	red blood cell
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
TE	tris-EDTA

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18

HLA-Antibody Determination

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18.1 Overview and Characteristics of Different HLA Antibody Detection Assays

Human leukocyte antigen (HLA) antibody detection is a key component in the evaluation of solid organ or allogeneic hematopoietic stem allograft recipients. The goal is to uncover HLA-specific sensitization due to prior pregnancies, transplantations, blood transfusions, or heterologous immunity induced by infections.

18.1.1 History and Evolution of HLA Antibody Detection Assays

The evolution of HLA antibody detection assays and their analytical principle are summarized in Figure 18.1. The first widely used assay for the detection of HLA antibodies was the complement-dependent cytotoxicity crossmatch (CDC-XM) [1]. In this assay, T- and/or B-cells of an individual are incubated with the serum of a patient. The binding of the patient's antibodies to the T- and/or B-cells is then made visible by complement-dependent cytotoxicity, leading to a color change of the affected cells. Instead of using only cells from one person, the cells from 30 to 60 different persons can be compiled in individual wells on a plate. The readout was the percentage of positive CDC-XM in the whole cell panel and was termed "panel-reactive antibodies" or "PRA" (e.g., if 10 of 60 reactions were positive, then the PRA was 17%). While the PRA assessment of class I HLA antibodies via T-cells was soon well established, the PRA calculation of class I and II HLA antibodies via B-cells was notoriously difficult and never gained wide clinical application.

In the early 1980s, the flowcytometric crossmatch was developed, which provided higher sensitivity to detect HLA antibodies (see also chapter on crossmatching). However, this method was not used to calculate PRA. Both the CDC and the flowcytometric crossmatch had the inherent limitation that they detected not only HLA antibodies but also many other – often irrelevant – antibodies. It became clear that an

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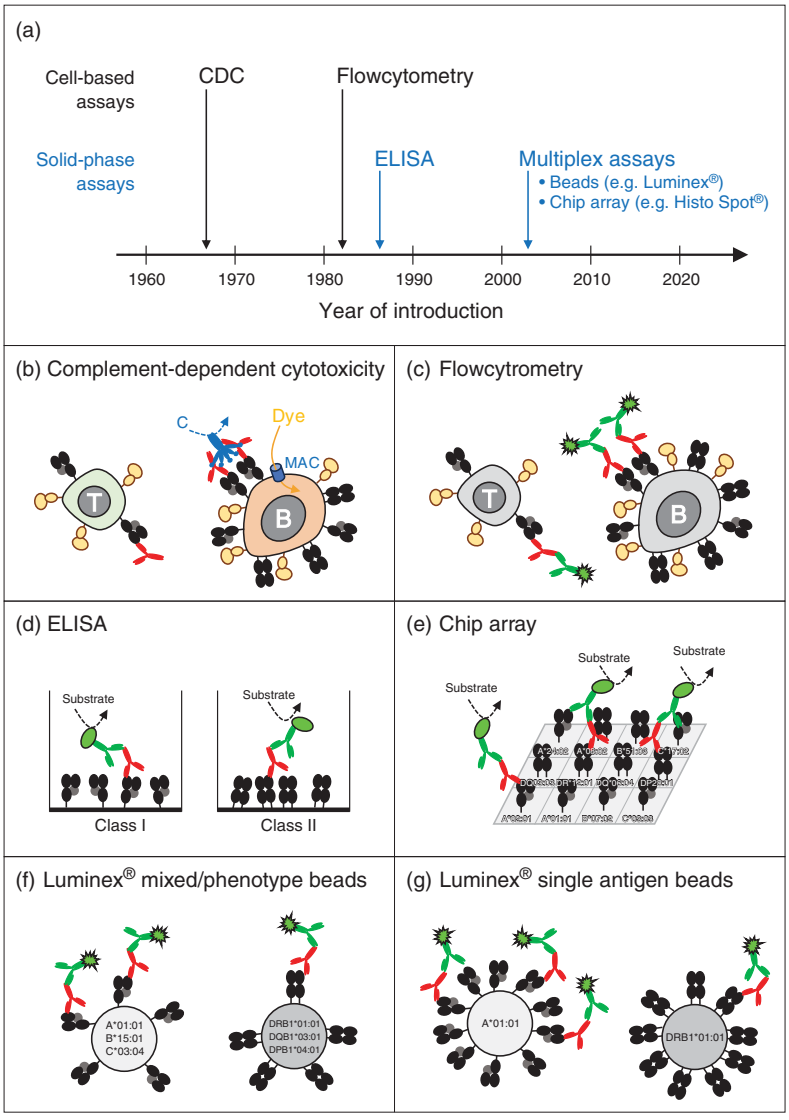

Figure 18.1 Evolution and the analytical principle of HLA antibody detection assays. (a) Year of introduction of different HLA antibody detection assays. (b–g) General analytical principle of the different HLA antibody detection assays. Red-colored antibodies refer to HLA antibodies in the blood of the patient. Green-colored antibodies refer to reporter antibodies. C1q as the initiating molecule of the complement cascade is colored blue in (b). Yellow surface structures in (b) and (c) represent non-HLA molecules. C = complement; MAC = membrane attack complex.

assay, which detects specifically HLA antibodies, would be a significant step forward. In the mid-1980s, HLA molecules were extracted from cells and coated onto ELISA plates as either a mixture of HLA molecules class I or HLA molecules class II. The readout was done by a standard ELISA method. The results were usually reported as

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one of four categories: (i) no HLA antibodies, (ii) HLA antibodies class I, (iii) HLA antibodies class II, and (iv) HLA antibodies class I and class II. Although this technology improved the sensitivity and specificity for the detection of HLA antibodies, it did not allow us to define against which HLA alleles the antibodies were directed.

In the early 2000s, this problem was resolved with the introduction of multiplex assays (i.e., bead array or chip array). Especially the bead array platform Luminex® evolved rapidly to the new standard method for HLA antibody detection. In this assay, up to 100 different beads per HLA class were covered with either one single HLA allele (i.e., single antigen beads) or a full HLA class I or II set from one individual (phenotype beads) [2]. The single antigen bead assays on the Luminex® platform and the newest generation of the single antigen chip array assays (Histo Spot®) allowed us for the first time to detect the specificity of HLA antibodies on the allele level with high sensitivity.

18.1.2 Advantages and Limitations of Different Assays

Today, cell-based assays to screen for HLA antibodies are mostly replaced by solid-phase assays. The key advantages and limitations are summarized in Table 18.1. Depending on the financial resources and the risk stratification concept of the transplant center, screening for HLA antibodies is performed either in a stepwise approach or directly with the most-informative but also the most-expensive single antigen assays. In the former approach, less-expensive "screening" assays (ELISA or bead array) are used to select patients having HLA antibodies, which are subsequentially specified by single antigen assays.

18.1.3 Current Status of HLA Antibody Detection Assays

Single antigen bead assays must be considered a major breakthrough in HLA diagnostics and transplantation medicine. In combination with advanced high-resolution HLA typing including all loci, two very important applications became reality that were already envisioned decades ago. First, the assessment of donor-specific HLA antibodies can be performed virtually without using any cells (i.e., virtual crossmatch). Second, the broadness of sensitization – and hence the probability of finding a donor organ without any donor-specific HLA antibodies – can also be performed virtually by comparison of the HLA antibody profile of the patient with large sets of HLA-typed persons in a population (i.e., virtual population-reactive antibodies [vPRA]). These two tools significantly improved pretransplant risk stratification algorithms and prioritization rules for waitlisted patients. In addition, they enabled successful national and international paired kidney donation programs.

Cell-based assays still have value in the final compatibility evaluation (see crossmatch section), but solid-phase assays replaced them for HLA antibody detection purposes. As mentioned previously, the Luminex® single antigen bead assay has emerged as the standard for HLA antibody detection in most HLA laboratories, and we will therefore describe this assay in more detail in the following section.

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Table 18.1 Summary of major advantages and limitations of different HLA antibody detection assays.

Assay	Advantages	Limitations
CDC	• Cheap • The natural configuration of HLA molecules on the cell surface • Detects complement-binding HLA antibodies	• Low sensitivity • Low specificity for HLA antibodies • Determination of HLA antibody specificity very difficult, especially for class II
Flow cytometry	• Cheap • The natural configuration of HLA molecules on the cell surface • More sensitive than CDC	• Lower sensitivity than Luminex® or chip array-based assays • Low specificity for HLA antibodies • Determination of HLA antibody specificity very difficult
ELISA	• Cheap • Specific for HLA antibodies • Automated high throughput	• Limited sensitivity • Determination of HLA antibody specificity very difficult • Denatured HLA molecules
Chip array	• Specific for HLA antibodies • High sensitivity • Precise determination of HLA antibody specificity • Automated high throughput	• Expensive • Denatured HLA molecules • Complex analysis • Limited literature to guide clinical use
Luminex® mixed	• Low cost • Specific for HLA antibodies • Automated high throughput	• Lower sensitivity than Luminex® single antigen beads • Determination of HLA antibody specificity difficult
Luminex® single antigen beads	• Specific for HLA antibodies • High sensitivity • Precise determination of HLA antibody specificity • Extensive literature and evidence to guide clinical use • Can be used for eplet mapping	• Expensive • Denatured HLA molecules • Complex analysis

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18.2 The Multiplex Bead Array Platform (Luminex®)

18.2.1 Introduction to the Platform

The Luminex® single antigen bead assay uses a panel of polystyrene beads, which consists usually of about 100 beads per HLA class. Each of these beads is coated with HLA molecules of only one specificity and contains a combination of two fluorochromes of different fluorescent emission intensities that are embedded within the beads. The latter allows us to obtain a unique fluorescent emission signal of each bead.

Currently, single antigen beads are sold by two vendors (LABScreen™ Beads by OneLambda® and LIFECODES beads by Werfen®). Beads of these two vendors differ in bead panel composition with respect to the specificities of HLA molecules coated onto the beads as well as bead properties. Differences in the bead properties concern, among others, the origin of the HLA molecules as well as their density on the beads and can influence the assay results [3–5]. For practical reasons, it is therefore important to be familiar with the characteristics of the beads from the vendor used in the respective HLA laboratory.

To perform the assay, patient serum is incubated with the bead mix, which allows the HLA antibodies in the serum to bind to the bead carrying the target HLA molecules. Following washing steps, a phycoerythrin-labeled antihuman IgG secondary antibody that binds to the HLA antibodies captured on the beads is added. The Luminex® instrument contains two lasers that are required to generate the readout of the assay. The red laser (excitation at 635 nm) excites the fluorochromes in the bead, leading to a unique fluorescent emission signal, which allows the identification of the specificity of the bead. The green laser (excitation at 532 nm) excites phycoerythrin of the secondary antibody and thereby enables the determination of whether an HLA antibody has bound to the specific HLA molecule on the bead or not. Figure 18.2 summarizes and visualizes the principle of the Luminex® single antigen bead assay.

The main readout of the assay is the level of fluorescence expressed as the mean fluorescence intensity (MFI). Such MFI values give an estimate of the relative amount of bound HLA molecules under the specified test conditions, even though certain technical issues need to be considered (see the following paragraph).

In the Luminex® single antigen bead assay, several controls are always taken along: (i) a negative control bead (with no HLA molecules on the bead), (ii) a positive control bead (PCB)(coated with human IgG), (iii) a negative serum, and (iv) a positive serum. Depending on the vendor's instructions, MFI values can be adjusted by taking the MFI of the negative control bead and the negative serum into account. Another approach is to subtract the average MFI values of the beads carrying the self-HLA of the recipient, which is likely biologically more correct [6].

18.2.2 Technical Challenges and Limitations

While the performance of the single antigen bead assay is straightforward, the determination of a positive or negative result can be challenging. Besides the definition

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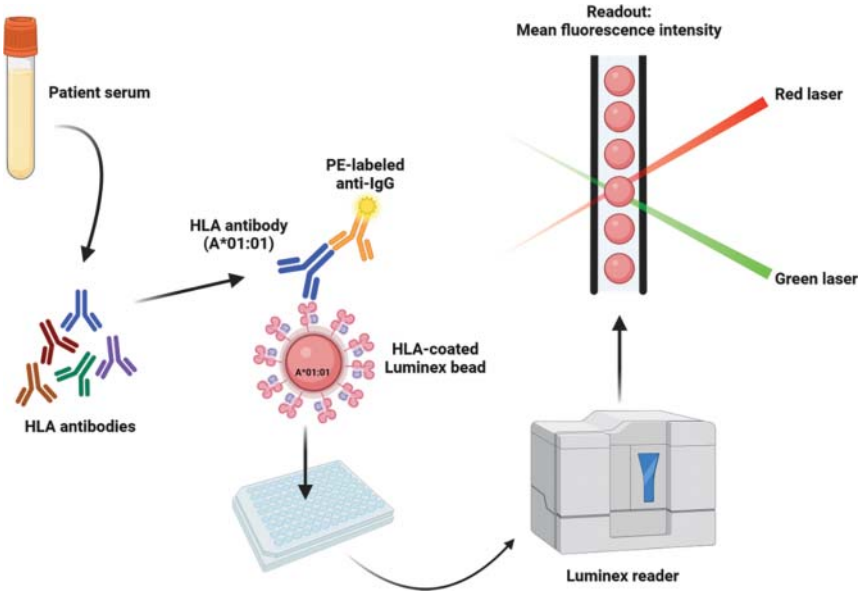

Figure 18.2 The principle of the Luminex® single antigen bead assay. Patient serum containing HLA antibodies is incubated with single antigen beads. If the respective HLA antibody (here anti-A*01:01) is present, it binds to the bead carrying the target HLA molecule. Subsequently, a phycoerythrin (PE)-labeled anti-human IgG secondary antibody is added. The Luminex® instrument allows for the detection of the respective bead (red laser) and the respective HLA antibody (green laser). The intensity of the signal is measured as MFI. Created in BioRender.

of a clinically meaningful cutoff (see Section 18.2.5), several technical issues as well as limitations need to be considered, which will be discussed in the following paragraphs. Figure 18.3 provides an overview of the technical aspects that may influence the assay readout.

18.2.2.1 Technical Caveats Leading to False-Negative Results or False Low MFI

Complement Interference The presence of high-titer complement-activating HLA antibodies may hamper the detection of HLA serum antibodies in an SAB assay. Notably, not all HLA antibodies are able to initiate the complement cascade to the same extent. It is nowadays well known that mainly the IgM and IgG isotypes and, among the latter, predominantly the subclasses IgG1 and IgG3 are capable of binding complement. The deposition of complement-split products (such as C1q and C3) can lead to steric hindrance of binding of the secondary antibody, thereby causing a false-negative result (Figure 18.3a) [7]. Several approaches with the goal of eliminating complement or preventing its activation exist. These include pretreatment of the serum sample with heat, dithiothreitol, or ethylenediaminetetraacetic acid (EDTA) [8]. Another option is to use plasma instead of serum samples, which has together with the addition of EDTA to serum samples become the mainly used strategy in many HLA laboratories to overcome the problem [9].

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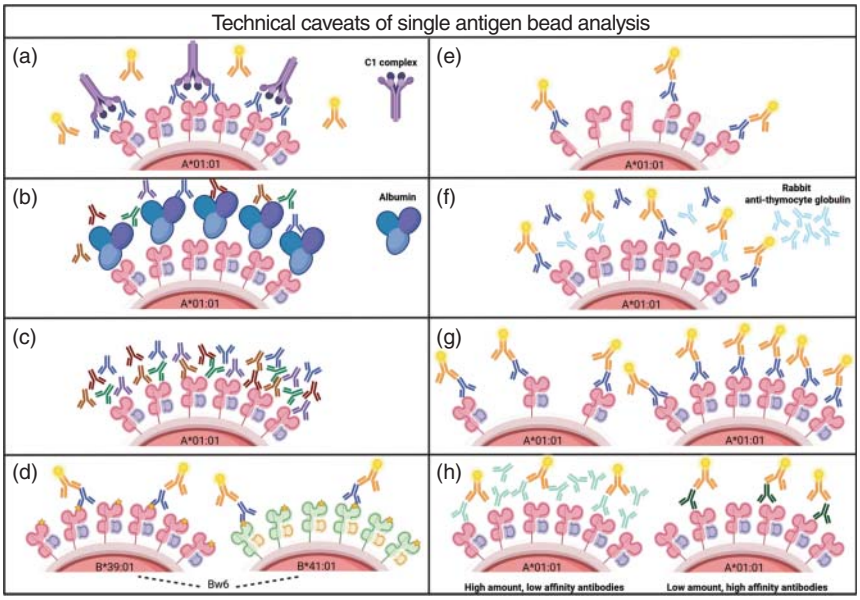

Figure 18.3 Technical caveats of single antigen bead analysis leading to false-negative (a–d) or false-positive results (e–h). (a) Deposition of complement leads to steric hindrance of binding of the secondary antibody. (b) Serum proteins (here albumin) mask the binding of the HLA antibodies of interest. (c) Oversaturation of the investigated HLA antibodies leads to steric hindrance of their binding to the single antigen beads. (d) HLA antibodies targeting a public epitope (here Bw6) spread among different single antigen beads carrying the respective epitope. (e) Denatured HLA molecules expose "cryptic" epitopes for the binding of HLA antibodies. (f) Rabbit anti-human HLA antibodies bind to the single antigen beads and can be detected by the secondary antibody. (g) Antigen density influences the MFI in the single antigen bead assays. (h) Antibody concentration and affinity influence the MFI in the single antigen bead assay. Created in BioRender.

Interference of Other Serum Components In addition to complement components, other serum proteins (such as albumin, haptoglobin, and transferrin) may interfere with the assay by masking the binding of the HLA antibodies of interest (Figure 18.3b) [10]. It has also been described for HLA antibodies of the isotypes IgM and IgA that interference with HLA antibodies of the IgG isotype can occur, which consequently leads to a decrease in the measured IgG signal [11].

The Prozone or High-Dose Hook Effect While the term prozone or high-dose hook effect has been for many years erroneously used for the phenomenon of complement interference [12], it basically refers to the fact that diluted serum may sometimes result in higher MFI values than undiluted serum. The reason for this false low signal is an oversaturation of the investigated HLA antibodies that leads to steric hindrance of their binding to the SAB (Figure 18.3c). Notably, dilution can diminish both the prozone effect and complement/serum component interference, resulting in higher MFI values. In certain cases, this approach can uncover the real extent of HLA sensitization. Additionally, it underlines that there

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is no linear correlation between MFI values as measured in the SAB assay and the concentration of HLA antibodies in vivo [13].

Binding to Public Epitopes A public epitope is an epitope that is shared by multiple antigens [14]. HLA antibodies that target such a public epitope shared by multiple HLA specificities will bind to more than just one single antigen bead (Figure 18.3d). This can lead to the distribution of the signal with a consequently lower MFI, underestimating the strength of the HLA antibody. Notably, such a signal distribution is not a real false-negative result but requires consideration of the HLA epitope concept [15, 16].

18.2.2.2 Technical Caveats Leading to False-Positive Results or False High MFI

Denatured HLA Molecules Resulting from the manufacturing process by recombinant technology, SAB may express denatured molecules on their surface. For class I molecules, such a denaturation can result in lacking of β2-microglobulin or parts of the transmembrane region or cytoplasmic tail being exposed to the surface, while these structures are anchored in the cell membrane in vivo. This may expose usually not accessible so-called cryptic epitopes for binding of antibodies in the SAB assays (Figure 18.3e). Such "natural" antibodies have first been described in nonimmunized males against modified structures of rare HLA molecules but were meanwhile also found against more frequently existing HLA types [17–19]. Even though still a matter of debate, the clinical relevance of these antibodies is likely limited [20–22].

As a consequence of the detection of these antibodies, one manufacturer has temporarily released the so-called ibeads, which largely expressed native HLA molecules. Since these beads are no longer available, repeated testing following acid treatment of SAB can be used to confirm the presence of "natural" antibodies. More practically, it is useful to identify problematic beads for every lot used by analyzing SAB results of nonimmunized males in order to better recognize such false-positive results [23].

Interference of Medication Whenever a patient has been treated with polyclonal anti-thymocyte globulin before SAB testing (the half-life of an IgG antibody is about 25 days), a false-positive result should be regarded as possible. The reason is that rabbit anti-human HLA antibodies can bind to the beads and can also be partly detected by the anti-human IgG [24, 25] (Figure 18.3f). Notably, a signal-lowering effect might also be possible since competition for SAB binding between rabbit anti-human HLA antibodies and human HLA antibodies can occur.

False-positive reactions due to the application of intravenous immunoglobulin are generally less likely or effects are small because such products are usually pooled from many plasma donors, leading to the dilution of HLA antibodies potentially present [26].

Antigen Density, Concentration, and Antibody Affinity All factors can influence the MFI signal in an SAB assay (Figure 18.3g, h) and are described in Section 18.3.1.1.

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18.2.3 Determination of a Positive Result

Ideally, a set MFI cutoff to determine a positive result should define clinically relevant HLA antibodies. However, even though many studies have clearly established the clinical relevance of donor-specific HLA antibodies (DSA) detected by an SAB assay [27, 28], there is currently no consensus within the transplant community on what MFI cutoff value should be used to consider an HLA antibody as relevant.

The reason for the lack of consensus is that the antibody strength, for which the MFI value gives an estimate, is only one of the many factors determining the pathogenicity of HLA antibodies. On the individual patient level, the missing full understanding of when a DSA will lead to antibody-mediated rejection (ABMR) can be emphasized by the fact that some patients transplanted in the presence of pretransplant DSA show uneventful clinical courses, while other patients present with early ABMR [29].

Thus, it is recommendable that HLA laboratories set their own MFI cutoff in close collaboration with the clinicians and in the context of a general risk stratification concept of the respective transplant centers. Importantly, a rather low cutoff (e.g., MFI 500 or 1,000) will lead to the identification of more patients having DSA by virtual crossmatching than a higher cutoff (e.g., MFI 3,000 or 5,000). While the first approach entails the risk of falsely defining a donor–recipient pair as "HLA incompatible" and, therefore, either limiting access to transplantation or applying unnecessary high immunosuppression to the patient, a higher cutoff may increase the risk for AMBR by missing relevant HLA antibodies.

Instead of using a uniform cutoff, another useful approach might be to individualize the decision on what HLA antibodies should be regarded as relevant. In this context, several aspects are worth considering: (i) Whenever possible, the HLA genotype of all loci of previous HLA sensitizing events should be obtained. Besides the HLA typing of the donor of the former transplanted graft, the typing of the child's father in women with a history of pregnancies can provide a more complete assessment of the sensitization history. For example, a rather low MFI of an HLA antibody directed against a specificity to which a woman had been exposed during pregnancy warrants caution and might be interpreted as relevant, even though the MFI is below a predefined cutoff. (ii) By contrast, HLA antibodies in previously nonimmunized individuals must be critically evaluated and preferentially analyzed on the eplet level in order to check for the plausibility of the results. In addition, as explained in Section 18.2.2.2, lot-specific variations exposing denatured HLA molecules should be taken into account. (iii) If an individual's HLA antibody profile reveals an eplet pattern, MFI values might be lower due to the distribution of antibody binding among the different SAB sharing the respective eplet. In such cases, highly relevant antibodies might be missed by a predefined cutoff.

Taken together, the determination of a positive SAB result is complex and requires both experience and careful consideration of both technical and clinical aspects.

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18.2.4 Modifications of the Generic Single Antigen Bead Assay

The generic SAB assay can be modified in several ways. One modification, the Rapid Optimized SAB (ROB) protocol introduced by Liwski et al., shortens the assay time course by slightly changing certain reagent volumes, and by decreasing incubation and spinning times [30]. These modifications yielded similar results to those achieved by the standard SAB protocol. The same group further developed an SAB test free of complement-inferred negative results, termed dual-antibody rapid test (DART) [31]. This test restores signals that are often indirectly lost in the normal SAB assay due to complement deposition, by additionally applying a PE-conjugated anti-C3d in the secondary antibody-binding step.

While the mentioned modifications may serve to shorten the assay time and circumvent false-negative results, other SAB assay protocol changes attempt to better predict the immunological risk of pre-existing or de novo DSA. To examine the isotype or subclass distribution of HLA antibodies, the generic PE-labeled anti-IgGpan can be simply replaced with PE-labeled anti-IgM [32], or anti-IgA [33], or with IgG-subclass-specific secondary antibodies [34]. To adequately adjust the cutoff in these alternative assays, multiple negative control sera should be applied as each secondary antibody exhibits a slightly different background signal signature in the SAB assay [34]. Furthermore, isotype- or subclass-specific monoclonal reference antibodies should be used as appropriate positive controls. Of note, the PCB, a basic component of the (IgG-detecting) SAB kit, will not serve longer as internal positive control upon testing of antibodies different than IgG. To overcome this limitation in the IgM assay, a commercially available IgM-specific PCB can be added to the SAB panel.

To better assess the harmfulness of pre-existing or de novo DSA with regard to their main effector functions, the SAB panel can also be used to examine the binding of complement split products (e.g., C1q [35], C3d [36], or C4d [37]). Since HLA antibodies mainly consist of IgG1 and IgG3 [38], their capability to induce complement binding is commonly high [39]. It is therefore not surprising that many studies found a strong correlation between the MFI values of DSA and their capability to bind the mentioned complement factors [40–43].

A study in which DSA subclass composition and their effector functions were investigated simultaneously revealed that high quantities of IgG2 and/or IgG4 can inhibit the effect of the subclasses IgG1 and/or IgG3 if the antibodies are directed against the same epitope. By contrast, IgG2 and IgG4, although per se not complement-activating, can act synergistically if their targeted epitopes are in proximity to IgG1/3-targeted structures on the same HLA molecule [44].

It should be noted that the results of the mentioned examinations must be interpreted cautiously as HLA molecules coated on SAB are rigid compared to mobile HLA molecules embedded in a cell membrane. Consequently, questions such as "Is the given DSA indeed activating the complement cascade?" or "Is the given DSA indeed leading to a crosslinking of bound Fc receptors to effectively induce intracellular signaling?" can still only be clarified by performing functional assays with cells, not SABs.

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Instead of a single measurement with undiluted serum, binding to SAB can also be investigated with several serum dilutions. The resulting SAB binding curve and the titer obtained (fold dilution of the respective serum that still reveals a positive signal) are mainly dependent on the quantity and apparent affinity of HLA antibodies present and consequently provide information on the overall potency of the tested HLA antibodies. The subject is further discussed in Section 18.3.1.2.

18.2.5 Assessment of the HLA Antibody's Targeted Epitope/Eplet

Originally, the SAB assay was designed to assess the HLA specificities of the examined recipient's HLA antibodies and ultimately their donor specificities on the basis of recipient/donor HLA typing information. Today, the SAB panel is increasingly used to identify functional subunits of the epitope, the so-called eplets, which are targeted by HLA antibodies. The demonstration of a clear eplet specificity reinforces the certainty that the antibodies investigated are real and the SAB signal is not caused by confounding factors. Moreover, knowing the sort and amount of eplets targeted by preformed DSA can potentially help tobetter characterize the additional immunological risk for DSA-positive recipients.

18.2.5.1 Eplets: Functional Parts of Epitopes

The paratope of a B-cell receptor (BCR) or an antibody recognizes highly specific structures on non-self-proteins. The surface area targeted on antigens is proportional to the surface area of the complementarity determining regions (CDRs) that are involved in the binding interaction (maximally six; each representing a radius of about 3–3.5 Ångström (Å) [45, 46]). The amino acid composition and the 3D expression of these CDRs are dependent on mutations within V/D/J genes located on chromosomes 14/2/22. Upon antigen exposure, the coding sequences within certain segments of these genes alter dynamically, leading to an increased antibody affinity. Since not all CDRs are necessarily involved in a given antibody–antigen binding, the definition of the area of the entire epitope (between 500 and 900 square Å [47–50]) and the critically involved structural subunits is highly challenging. To date, there are only two reports that provide X-ray crystallographic data of HLA antibodies complexed with HLA [51, 52]. They confirm that indeed not all CDRs must be involved in the binding interaction. It was even shown that a difference of solely one amino acid between the donor and recipient HLA can be sufficient to induce antibody formation [53, 54]. On the other hand, it was demonstrated that at least three CDRs must contribute to the binding interaction in order to result in an affinity sufficient enough to induce an effector function (i.e., complement binding).

René Duquesnoy was one of the first HLA researchers who used the early established immunological principles of allo-recognition to define the functional parts of epitopes on HLA molecules, namely amino acid patches located on accessible areas that can be ideally targeted by a single CDR. He developed the HLAMatchmaker [55] software, an algorithm that can be used to define such patches on donor HLA, termed "eplets." The HLAMatchmaker software compares high-resolution HLA

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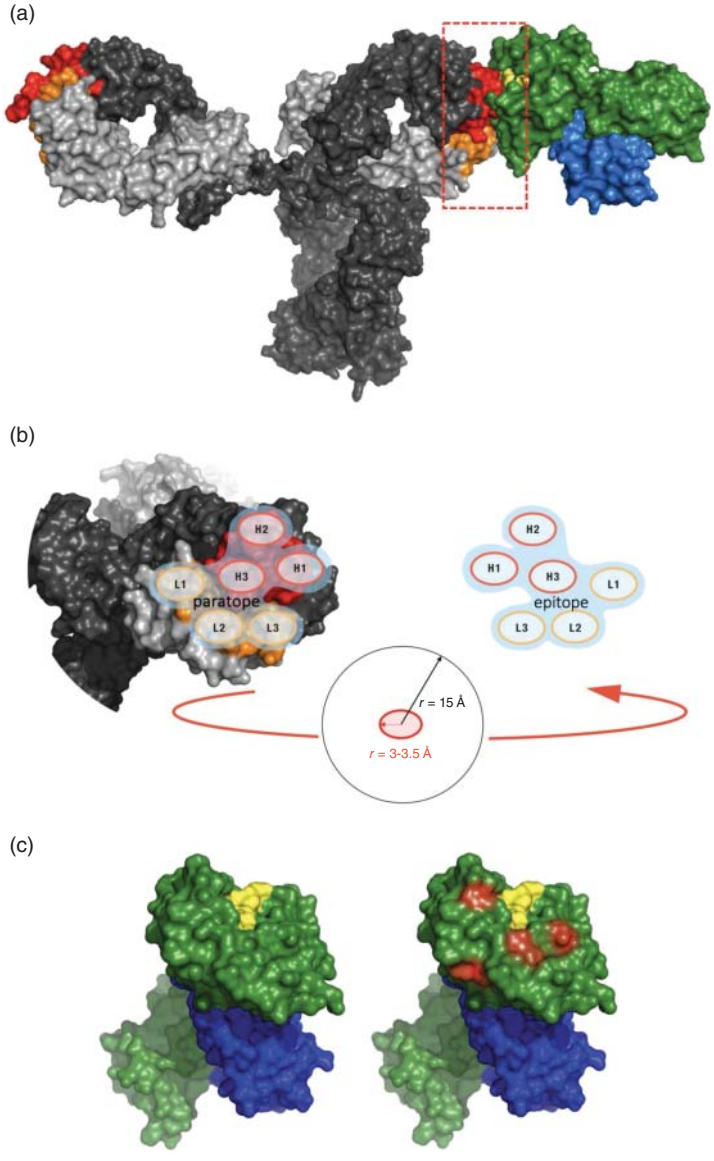

Figure 18.4 Size proportion of HLA/HLA–antibody complex, HLA epitope, and eplet.
(a) HLA-A*01:01/HLA–antibody complex with real size ratio. Black = heavy chains of antibody, gray = light chains of antibody, red = CDRs of heavy chain, orange = CDRs of light chain, green = heavy chain of HLA-A*01:01, blue = ß2-microglobulin, yellow = peptide. Red-framed region = binding interaction between paratope and epitope. Modeling of the complex by using the X-ray crystallographic data published by [52]. (b) Left: side view of the paratope of an antibody consisting of three CDRs of the heavy chain (red; H1, H2, H3) and three CDRs of the heavy chain (orange; L1, L2, L3). Middle: dimension of an eplet (red inner circle; radius = 3–3.5 Å [0.3–0.35 nm]) and the entire epitope (black outer circle; radius = 15 Å). Right: surface area of the epitope, potentially targeted by the six CDRs. (c) Visualization of structural mismatches between recipient and donor HLA. Left: HLA class I molecule of the recipient. Right. HLA class I molecule of the donor with four indicated mismatched eplets (red spots).

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information of donor and recipient to subsequently define all eplets expressed in the given donor HLA repertoire.

An eplet is defined as the functional part of an epitope, namely the 3D structure consisting of 1–5 amino acids located on the accessible surface of an HLA molecule that is assumingly targeted by a CDR of the antibody's paratope (Figure 18.4). Epregistry.com [56] lists all currently defined eplets and their verification status [57]. The latter indicates whether an eplet has been only theoretically defined or with a reported eplet-specific antibody signature. Many eplets have been defined by HLA-specific monoclonal antibodies (mAbs) [58].

18.2.5.2 Determination of Eplet Specificity by SAB

When additionally feeding the algorithm with SAB assay results of a patient's antibody serum, DSA eplet specificities can be visualized. However, eplets are unevenly distributed on the HLA specificities represented in the SAB panel, and thus determination of DSA eplet specificities, especially from the serum samples of highly sensitized patients, can be challenging. Therefore, extra SAB panels with extended HLA specificities (i.e., ExPlex beads) are additionally available. To define the eplet specificities of a given DSA with greater confidence, an adsorption/elusion approach can be applied. Thereby, the relevant HLA molecule is used to solely adsorb the antibodies of interest. Subsequently, these antibodies are eluted followed by SAB re-analysis. For a long time, the HLA source for such adsorption/elusion consisted of cells or platelets. Today, commercially available magnetic beads (MagSort™) [59] representing HLA molecules of unique specificities are an ideal tool for this purpose.

18.3 Additional Methods/Tools

18.3.1 Determination of Concentration and Affinity of DSA

Information about the quantity of circulating DSA and their apparent binding strength will potentially enhance pre-transplant risk stratification as well as post-transplant monitoring.

18.3.1.1 MFI Signal from SAB Assay Is Impacted by Antibody Concentration, Antibody Affinity, and Confounder Factors

Although the SAB assay is not quantitative, MFI values obtained are often incorrectly considered synonymous with antibody concentration. However, the signals obtained by SAB assays are additionally influenced by the apparent affinity of tested antibodies. Concretely, a high amount of low-affinity antibodies can produce a similar signal as a low amount of high-affinity antibodies. This is illustrated in Figure 18.5 upon binding curves derived from the titrations of two HLA-specific mAbs that slightly differ in their affinity. The confounding factors that can additionally impact the final MFI signal are described in Section 18.2.2.

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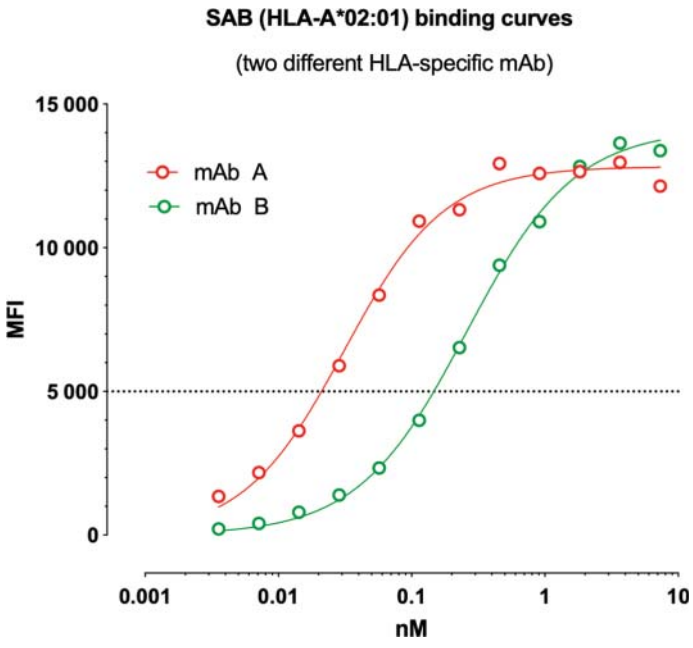

Figure 18.5 Antibody-binding curves demonstrating the impact of concentration and affinity on the MFI signal of the SAB assay. SAB-binding curves obtained from the titrations of two (red and green) HLA-A*02:01-specific mAbs. To reveal the same MFI (e.g., 5000; red dotted line), a lower concentration is needed for mAb A (red, clone SN230G6, eplet specificity 62GE) compared to mAb B (green, clone SN607D8, eplet specificity 144TKH), indicating a higher affinity for mAb A.

18.3.1.2 Serum Titration Partly Reveals HLA Antibody Concentration

To get an indication of the concentration of antibody present, patient sera can be diluted or fully titrated for SAB testing [60]. This labor-intensive and costly approach is often applied to monitor the efficacy of desensitization treatments [61]. The binding curve obtained from a serial dilution will reveal the titer of the tested antibody (the fold dilution needed still indicates a positive signal), as visualized in Figure 18.6. Of note, serum 3 reveals a high-dose-high-hook effect and/or a complement inference, leading to a reduced signal at upper concentrations. It is important to consider that the value of the titer is clearly impacted by antibody affinity as well. A high titer can, for instance, result from many antibodies of low affinity or from few antibodies of high affinity.

18.3.1.3 Definition of Antibody Affinity and Antibody Avidity

What is the definition of antibody affinity or antibody avidity? Affinity is the sum of the strength of all (noncovalent) interactions (hydrophobic interactions, hydrogen bonds, ionic bonds, and van der Waals interactions) between a single antigen-binding site on an antibody and a single epitope on the antigen. If an antigen expresses multiple identical epitopes or antigen molecules are present in appropriate proximity (e.g., HLA on SAB), the interactions between multiple

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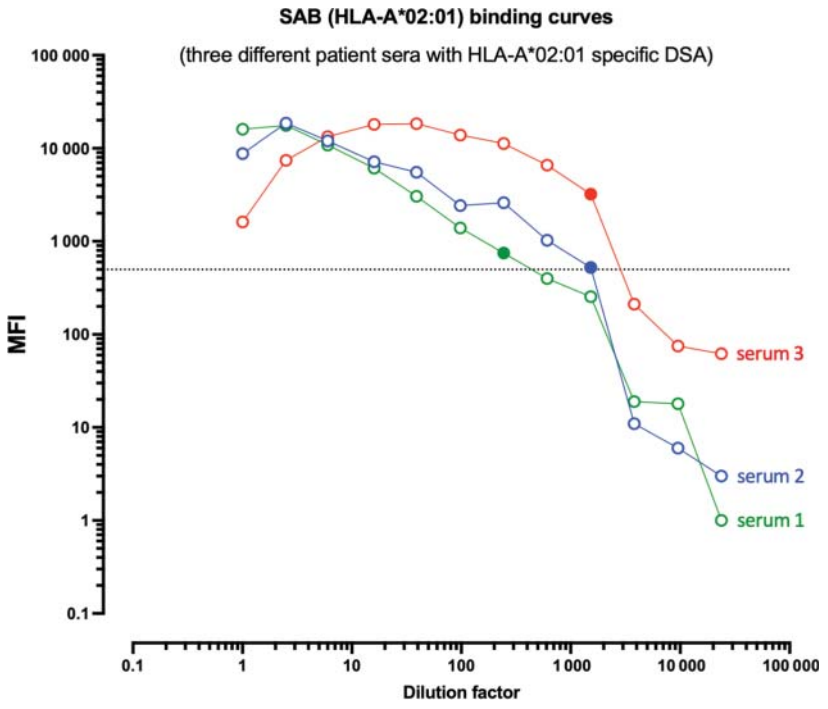

Figure 18.6 SAB-binding curves from dilutions of three patient sera. Binding curves of HLA-A*02:01-specific DSA from three different patients (green, blue, and red), using HLA-A*02:01-SAB. X-axis = concentration, y-axis = MFI of SAB test. The titer (dilution that still reveals a positive test signal = here a signal above the arbitrary cutoff of 500 MFI) of each DSA is indicated by the color-filled circle. The dotted line indicates the cutoff. Of note, the MFI result from undiluted serum is often not in accordance with the titer result, as the case here (signal ranking undiluted serum = green, blue, red; titer ranking = red, blue, green). DSA from serum 3 (red) demonstrates a pronounced high-dose high-hook and/or a complement interference effect.

antigen-binding sites on the (multivalent) antibody and the multiple epitopes reveal a significantly increased overall binding strength. This is termed avidity.

And how is affinity or avidity reported? Generally, this is done by using the dissociation equilibrium constant (K_D; [M]). For antibodies, the value of this constant, the K_D-value, represents the antibody concentration at which half of the antibody-binding sites on the antigen is occupied at the equilibrium of the paratope–epitope-binding reaction. Figure 18.7 visualizes the relationship between affinity and avidity by comparing the obtained K_D results from the interaction of HLA-A*02:01 with mAb W6/32 between four different measuring technologies, namely three solid-phase assays (with antigen coated to the solid phase) and one in-solution assay (where both binding partners, soluble HLA and the mAb, are interacting in free solution under near-native conditions).

SAB assays can be used to measure the binding strength of HLA-specific mAbs via titration; however, the results will indicate avidity, not affinity [62]. This is due to the high density of HLA molecules coated on SAB, leading to multivalent binding

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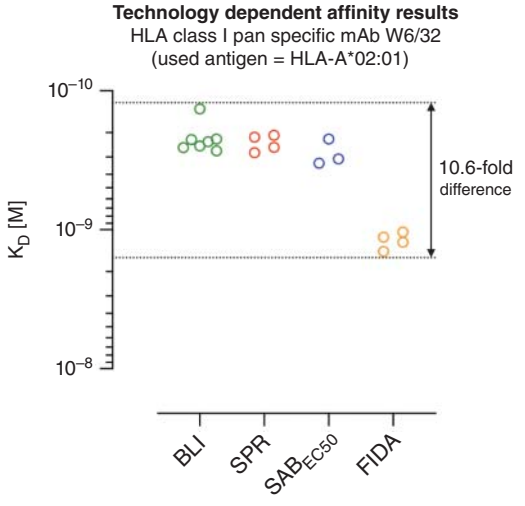

Technology	k_a [1/s]	k_d [1/Ms]	K_D [M]	CV	n
BLI	$2.76*10^5$	$7.21*10^{-5}$	$2.61*10^{-10}$	0.20	7
SPR	$6.38*10^5$	$1.53*10^{-4}$	$2.40*10^{-10}$	0.12	4
SAB_{EC50}			$2.86*10^{-10}$	0.20	3
FIDA			$1.21*10^{-9}$	0.12	4

Figure 18.7 Technology-dependent affinity results. (a) Affinity results obtained by the indicated technologies. Green line: lowest K_D-value (highest affinity). Orange line: highest K_D-value (lowest affinity). (b) Table with obtained affinity (K_D)-constants, on- and off-rates, and calculated coefficients of variation (CV). While BLI, SPR, and SAB are solid-phase assays, FIDA is an in-solution assay and reveals K_D-values that are shifted by about one magnitude. BLI = bio-layer interferometry, FIDA = flow-induced dispersion analysis, ka = association rate = on rate, kd = dissociation rate = off rate, K_D = dissociation equilibrium constant = affinity, M = molarity (1 M = 1 mol/l), SAB = single antigen bead, SPR = surface plasmon resonance. Source: [62]/John Wiley & Sons/CC BY 4.0.

and to the rebinding effect [63]. The use of solid-phase assays (i.e., SAB) for accurate determination of antibody-binding strength in serum samples is highly problematic due to the described confounding factors.

18.3.1.4 State of the Art and Future of HLA Antibody Affinity Measurement

HLA antibody affinity is currently not routinely assessed for transplant risk stratification. First, methods to appropriately investigate crude samples (e.g., serum) are not yet suitable for routine use, and, second, no studies have been conducted yet to show the clinical relevance of HLA antibody affinity. However, knowing the affinity of the antibodies tested would be important if only to learn to what extent it influences the results of the routinely used binding assays.

Real-time interaction measurement is the most appropriate way to determine antibody affinity by determining the velocity of the on- and off-binding reaction. Alternatively, an end point measurement can be performed, namely an investigation after the binding reaction has reached the equilibrium. The latter procedure needs a titration of either the antigen or the antibody to generate a dose–response curve from

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which the half maximum binding concentration can be drawn to indirectly assess antibody affinity.

HLA-specific mAbs have been assessed for their affinities/avidities using several biophysical methods: surface plasmon resonance (SPR) [64, 65], bio-layer interferometry (BLI) [66], microfluidic diffusional sizing (MDS) [66], flow-induced dispersion analysis (FIDA) [62], and SAB assays [62]. However, only a few publications report data from patient DSA. To mimic the heterogeneous matrix of crude samples, Visentin et al. spiked HLA-specific mAbs into negative control serum for their SPR experiments [65], while Schneider et al. investigated real patient serum samples with MDS measurements [66], and another group from Basel [62] published DSA-binding strengths assessed by FIDA from 20 pretransplant patients.

The two latter methods enable a simultaneous determination of antibody concentration and antibody in-solution. Here, both binding partners (i.e., soluble antigen and antibody) interact under near-native conditions (meaning that none of the binding partners is immobilized to a solid phase), which facilitates a higher accuracy of results. In-solution measurements were first used to characterize SARS-CoV-2 antibody concentration and affinity in patient sera [67]. The study showed that a significant proportion of the test samples represented an antibody concentration too low for accurate determination of the affinity. Since HLA antibody serum concentrations seem to be in a similar range [62], only a part of potentially interesting transplant patient samples may be testable by the current assay. However, the new method has a high potential for monitoring the concentration and affinity of DSA from hypersensitized patients, especially for testing the efficacy of desensitization treatments, and thereby could overcome the high costs for titration studies.

18.3.2 HLA Antibody Detection Beyond Analysis in Blood

The Luminex® SAB assay is well-established for the detection of HLA antibodies in serum and plasma. This raises the question of whether the same technique can also be used for the detection of HLA antibodies in another matrix.

In this regard, it has been tried to not only search for HLA antibodies in the serum of the patient but also determine whether DSA has homed to the graft. In situ detection of DSA could potentially discriminate harmful from less harmful DSA and serve as a marker for ABMR. Technically, an elution protocol on nonfixed kidney biopsy cores was applied [68, 69]. Using this elution protocol, the presence of DSA in the graft was associated with the extent of ABMR typical lesions as well as short-term graft outcomes in one study [68–70]. However, data on intragraft DSA detection are still scarce and the technique has not been implemented for routine diagnostic use yet.

In recent years, the HLA-specific memory B-cell compartment has been another focus of interest. In order to understand the potential significance of memory B-cells in transplantation, it is important to briefly recapitulate the generation and role of these cells. Following naïve B-cell activation upon recognition of the alloantigen via the BCR, maturation of these activated B-cells in the germinal center of secondary lymphoid organs gives rise to both plasma and memory B-cells. Long-lived plasma

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cells subsequently move to the bone marrow where they spontaneously produce HLA antibodies. These antibodies can then be routinely detected by SAB assay. However, unlike plasma cells, memory B-cells remain "silent" while circulating between the secondary lymphoid organs and the peripheral blood. Once they encounter the same HLA, memory B-cells can differentiate into antibody-producing cells, whereby they contribute to the serum HLA antibody repertoire. Consequently, SAB assay in the serum may only provide an incomplete picture of the humoral alloimmune response. Therefore, several assays to detect HLA-specific memory B-cells have been developed [71]. Besides the staining of these cells by HLA tetramers and subsequent detection in flow cytometry [71–73], mainly enzyme-linked immunospot (ELISpot) assays seemed promising [74–76]. However, these assays are labor-intensive and difficult to standardize. Therefore, there is now a main interest in the qualitative analysis of memory B-cell-derived HLA antibodies in the supernatants of B-cell cultures in vitro [77]. Figure 18.8 shows the principle of this memory B-cell assay. Without the need to sort B-cells, peripheral blood mononuclear cells are in vitro polyclonally stimulated and cultured for 10 days. The culture supernatants, containing memory B-cell-derived HLA antibodies, are subsequently harvested and IgG is isolated and concentrated. The obtained small-volume eluate is ready to be analyzed by SAB assay, which subsequently allows for a direct comparison with the serum HLA antibody profiles. Experiences with this method clearly show that HLA antibody profiles in serum and in culture supernatants are not identical. The question of whether the additional information on the HLA-specific memory B-cell compartment is able to

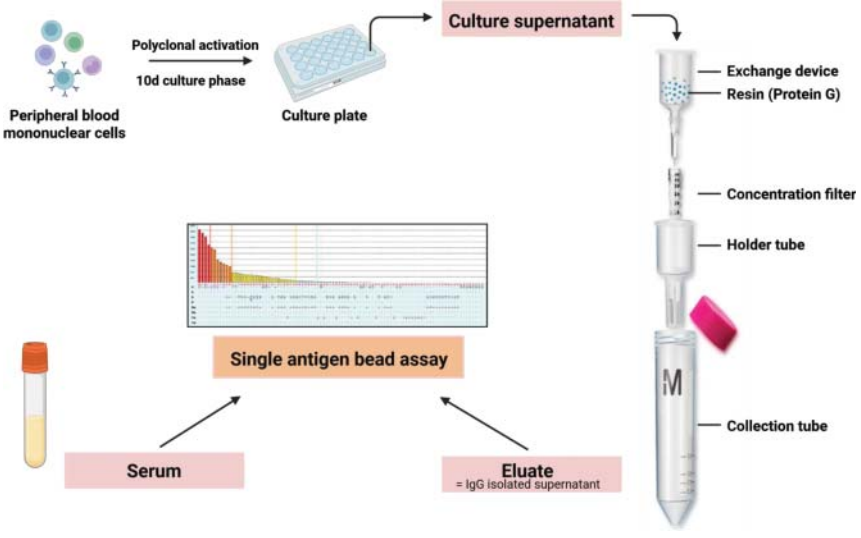

Figure 18.8 The principle of detection of HLA-specific memory B cell-derived antibodies in B-cell culture supernatants. Peripheral blood mononuclear cells are polyclonally stimulated for 10 days. The culture supernatant is harvested and processed by a protein G-based IgG affinity purification and concentration method. The obtained eluate (=IgG isolated supernatant can be analyzed by single antigen bead assay, which allows for a direct comparison with the serum single antigen bead assay results). Created in BioRender.

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supplement and ultimately refine the current immunological risk assessment has so far only been addressed in two clinical studies that showed conflicting results [78, 79].

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19

Low/Medium-Resolution HLA Typing

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19.1 Introduction

HLA typing is a mandatory test for the selection of donors and recipients for solid organ and hematopoietic stem cell transplantation. Additionally, it is well established that specific HLA class I and class II alleles are associated with predisposition or protection to different diseases including autoimmune diseases, infectious diseases, and cancer. Other areas of application of HLA typing are pharmacogenomics as HLA genotype determines severe hypersensitive reactions to certain drugs and vaccine development as response to antigenic peptides depends on their presentation by patient's HLA molecules. Considering these important clinical applications, different methods for HLA typing have been developed and implemented in routine laboratory practice.

Generally, these methods are classified into cellular, serological, and DNA-based methods. Genetic tests for HLA typing have different levels of resolution: low resolution defines the first field of WHO HLA nomenclature group of alleles equivalent to serological typing; high resolution defines the second field of WHO HLA nomenclature corresponding to the groups of HLA alleles with the same protein sequence within the antigen-binding site; and allelic resolution defines all of the field according to the WHO HLA nomenclature corresponding to individual alleles. Although in the last decade HLA high-resolution typing has been performed by many histocompatibility laboratories, low-resolution methods are still used mainly for typing of donors and recipients for solid organ transplantation and some disease associations.

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19.2 Cellular and Serological Tests for HLA Typing

Cellular tests, such as mixed lymphocyte reaction [1], homozygous cell typing (HCT) [2, 3], and primed lymphocyte typing (PLT) [4], had limited application for HLA class II typing. Mixed lymphocyte reaction identifies HLA class II discrepancies between the donor and the recipient, their allogenic impact, and permissiveness [5]. In MLR tests, lymphocytes from the recipient and the potential donor are incubated in vitro, and the proliferative response of lymphocytes as a result of HLA mismatches is detected by the incorporation of radioactively labeled nucleotides in the synthetized DNA. The inactivation of donor's lymphocytes allows to measure the proliferative responses of recipient's lymphocytes. However, such techniques were very labor intensive and difficult to standardize.

The serological tests have been used for a long time for routine HLA typing in histocompatibility laboratories. The most commonly applied method was complement-dependent cytotoxicity test (CDC) or micro lymphocytotoxicity test developed by Terasaki and McClelland [6]. In this test, HLA molecules expressed on lymphocytes are assessed by incubation with anti-HLA antibodies with known specificity and complement. If the HLA molecules are recognized by the antibody, the Fab fragment of the antibody binds to the epitope, and the antigen–antibody complexes activate the complement, which results in lymphocyte lysis. To differentiate lysed from live cells, vital dyes such as eosin dye or fluorescent dye that selectively stain dead and live cells are used. Positive results are assigned by microscope reading of the plates if more than half of the cells in the well are killed. Most laboratories use numeric scores from 0 to 8 to assign the percentage of killed cells per well. As a first step, this method requires the isolation of viable lymphocytes from peripheral blood, and in the case of typing of deceased donors, it requires the isolation of viable lymphocytes from lymph node or spleen, by gradient centrifugation. Considering the differential expression of HLA molecules, HLA class I typing is performed on unseparated PBMC or T cells, while HLA class II typing requires isolation of B cell suspension. T cells and B cells could be separated from peripheral blood via magnetic beads, coated with T-cell- or B-cell-specific antibodies. Initially, the typing reagents were sera with well-defined HLA specificities from multiparous women. Such sera were exchanged by the laboratories within International HLA and Histocompatibility Workshops, and the first serological HLA specificities were defined [7]. Later, monoclonal antibodies recognizing HLA broad or split specificities recognized by WHO replaced human allo-antisera in the CDC test. Currently, the laboratories performing serological HLA typing use commercially available, well-standardized HLA typing kits with monoclonal or polyclonal antibodies against different HLA specificities distributed in microtiter plates. According to the EFI standards for histocompatibility and immunogenetic testing, at least two monospecific sera/antibodies or at least three non-overlapping multispecific sera are used to define each HLA-A and B antigens, and at least three monospecific or five partially non-overlapping multispecific sera are used to define each HLA class II antigen. Each typing tray must also contain a positive control serum, reacting with cells expressing HLA class I or class II

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molecules, and a negative control serum without leukocyte reactive antibodies. The main advantage of the serological test is that only expressed HLA molecules are detected, and therefore, it could be used to distinguish HLA null alleles [8]. Additionally, serological typing can be performed quickly (in less than two hours) and is applicable for urgent tying of deceased donors. However, this test has several limitations. Due to the unavailability of specific antisera or antibodies, serological tests could not define the increasing number of HLA specificities [9] with clinical relevance. Additionally, due to the cross reactivity of antibodies or antisera used, split HLA antigens could not always be assigned unequivocally [1, 10, 11]. Serological typing was not always reliable for HLA-C, HLA-DR, and HLA-DQ antigens [1, 12, 13], and with the development of DNA-based methods, the high error rate was observed: 33% for HLA-C locus [12] and 25–27% for HLA-DR [13]. An additional limitation of serological HLA class II typing is the requirement to isolate enough viable B lymphocytes. Although proficiency testing studies using serology and DNA-based tests showed 7.1% misassignment for serological HLA-A typing [11] and 22% misassignment for serological HLA-B typing [10], serological typing for a long time has been used as a main method for HLA-A and HLA-B typing.

19.3 DNA-based Methods for HLA Low-Resolution Typing

Different molecular methods have been developed and introduced for HLA typing replacing serological tests in routine diagnostics. DNA-based methods are not restricted by the requirement to use live lymphocytes and synthetic reagents and could be easily standardized and modified as new HLA alleles are discovered. One of the first molecular methods used for the analysis of the HLA class II was restriction fragment length polymorphism (RFLP) [14, 15]. This method was based on the fragmentation of genomic DNA with restriction endonucleases, electrophoretic separation of the fragments with different lengths, transfer on nitrocellulose membrane, and hybridization with labeled DNA probes. Although the methods were labor intensive and time consuming and required a relatively high amount of DNA and knowledge on DR-DQ linkage disequilibrium to distinguish DR specificities with identical RFLP patterns, the early studies showed the important clinical potential compared to serological tests [16]. With the development of polymerase chain reaction (PCR), various methods have been developed and applied for HLA low- or high-resolution typing, including PCR-sequence-specific primers (PCR-SSP), real-time PCR-based methods, PCR-sequence-specific oligonucleotide probes (PCR-SSOP), Luminex-based bead array techniques, conformational-based methods such as reference strand-mediated conformational analysis (RSCA), PCR-sequencing-based typing (PCR-SBT), and next-generation sequencing [17–19].

Among these, the most used methods for low-resolution HLA typing are PCR-SSP, real-time PCR-SSP, PCR-SSOP, and Luminex-based assays. These methods use primers or probes to detect polymorphic sequences specific to HLA allele groups.

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19.3.1 PCR-SSOP Methods

PCR-SSOP (polymerase chain reaction–sequence-specific oligonucleotide probes) was one of the first PCR-based methods used initially for HLA class II and later for HLA class I genotyping. This test uses a hybridization of a panel of oligonucleotide probes specific for different HLA alleles or group of alleles with locus-specific amplicons containing the polymorphic exons of HLA class I (exons 2 and 3) and class II (exon 2 for DRB1 and exons 2 and 3 for DQB1) loci. Under stringent hybridization conditions, a stable binding between the probe and the complementary target DNA will occur. Initially, two types of dot-blot PCR-SSOP method have been developed – forward and reverse. In the forward PCR-SSOP, the locus-specific amplicons after denaturation are plotted on a solid surface membrane and are incubated with a solution containing a specific labeled probe. After hybridization and wishing steps, the substrate is added, and depending on the markers, positive reactions are determined. This test requires separate membranes for each specific oligonucleotide probe. HLA typing methods based on the forward dot blot were initially developed for HLA class II typing [20–22] and later for HLA class I typing [23–27]. Reverse PCR-SSOP is used more frequently in H&I laboratories. In this test, all oligonucleotide probes are attached to a membrane. The amplicon is labeled during PCR amplification using 5′ biotinylated primers. After denaturation, the labeled amplicon is incubated with a membrane on which different specific oligonucleotide probes are attached. Hybridization will occur only with the probes complementary to the sequence of the amplicon After the washing step, a horseradish peroxidase conjugated to streptavidin is added inducing a color change in substrate tetramethylbenzidine to detect specific hybridization patterns [28–30]. Automated and semiautomated typing systems that use the reverse PCR-SSOP principle were developed.

Currently, many laboratories use routinely reverse PCR-SSOP with multiplex bead-based array technology – Luminex xMAP technology [31]. The xMAP technology was initially applied for HLA-DQA1 typing by Fulton et al. [32] and later applied to other HLA loci [33]. Currently, commercial kits are available for HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DQB1, HLA-DQA1, HLA-DPB1, and MICA loci. The assays utilize polystyrene microbeads labeled with two fluorescence dyes that are excited by a red laser at 635 nm. The light emitted is detected in red (660 nm) and infrared (730 nm) using a Luminex flow cytometer. By measuring the emission intensities in these 2 channels, up to 100 different beads could be identified in Luminex 100/200 devices. LabScan3D flow cytometers allow 500 different beads to be used in one test. Similar to other reverse PCR-SSOP methods, HLA-specific probes covalently coated to the beads are used. The first step of the assay includes locus-specific amplification with biotinylated primers. Some of the commercially available kits (Immucor, USA) use asymmetric PCR and generate single-stranded amplicons, while the other assays (OneLambda, USA) use conventional PCR, followed by denaturation of double-stranded amplicons generated. Then, the locus-specific amplicons hybridize at a predefined controlled temperature with the mixture of beads, each coated with a probe with known HLA

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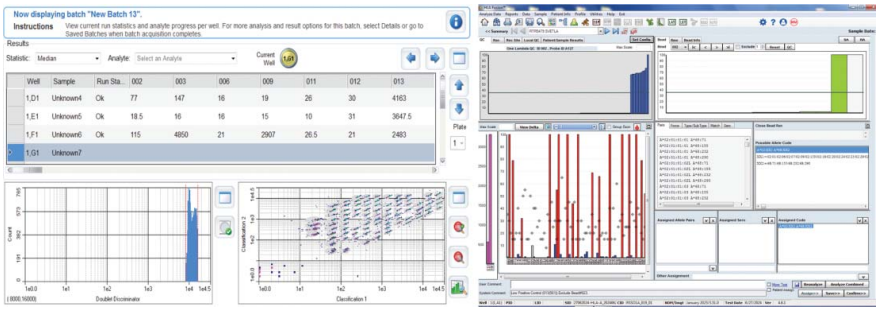

Figure 19.1 Luminex-based PCR-SSOP method. Luminex xMAP technology utilizes a multiplex bead-based array. The polystyrene microbeads are labeled with two fluorescence dyes. By measuring the emission intensities in 2 channels, up to 100 or 500 different beads could be identified in Luminex 100/200 and LabScan3D devices, respectively. Each type of bead is coated with probes, specific for different HLA alleles or group of alleles. The labeled locus-specific amplicons hybridize with beads and are stained by adding phycoerythrin conjugated to streptavidin. The green laser of Luminex machine detects phycoerythrin, while the red laser detects the fluorescent code of the beads. For each probe, positive or negative results are determined based on predefined mean fluorescence intensity (MFI) values: 1) xPONENT data collection software (Luminex); 2) HLA Fusion (OneLambda, Thermo Fisher Scientific, USA) software for data analysis.

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specificity. After washing steps, beads with bound amplicons are stained by adding phycoerythrin conjugated to streptavidin. The green laser of the Luminex machine detects phycoerythrin, while the red laser detects the fluorescent code of the beads. Each assay contains internal positive control for each of the exons amplified. Internal hybridization controls are used in the normalization of the values during the assessment of the reaction patterns. For each probe, positive or negative results are determined based on predefined mean fluorescence intensity (MFI) values that are specific to the lot number of the kit (Figure 19.1). Luminex-based assays have relatively high throughput allowing up to 96 different samples to be analyzed simultaneously for a given locus. They have a higher sensitivity and resolution compared to conventional PCR-SSOP methods and low to intermediate resolution. High-resolution kits were developed for the 500-beads system LabScan 3D, allowing typing of common and well-documented HLA alleles [34].

The main advantages of PCR-SSOP methods are that they require a small amount of genomic DNA, can be fully or semiautomated, and have mid-throughput. The methods have limited capacity to resolve ambiguities due to cis- and trans-polymorphisms. PCR-SSOP requires two steps: amplification and hybridization; therefore, it is not suitable for typing of deceased donors. The Luminex-based methods allow intermediate resolution, and many laboratories apply such technology for HLA typing of patients and donors for organ transplantation and disease association.

19.3.2 PCR-SSP Methods

PCR-SSP (polymerase chain reaction–sequence-specific primers) was designed by Newton et al. based on the amplification mutation refractory system (ARMS) [35]. This method uses a panel of primers specific for individual alleles or group of alleles for amplification. The principle of the method is that only primers with sequences complementary to the target DNA template will be used more efficiently in the first critical cycles of the PCR reaction than primers containing one or several nucleotide mismatches in their 3′ end. The amplification will take place only if the template DNA contains the target sequence of the primers, while noncomplementary primers will not bind to the DNA and no amplification will occur. The amplified fragments are determined by agarose gene electrophoresis (Figure 19.2). HLA specificity is determined based on the presence and the defined length of the amplicons for each primer mix. In addition to HLA-specific primers, each primer mix contains positive internal control to detect technical failures – primers amplify the conservative region of the genomic DNA. The PCR-SSP method was initially developed for HLA-DRB1 [36] and DQB1 genotyping [37, 38] and later for typing of HLA class I loci [39–43]. In order to increase the resolution and to reduce the number of primer mixes used, two-step nested PCR-SSP methods were developed [44–46].

PCR-SSP is a very rapid and reliable method and has been used in many laboratories for HLA low-resolution typing in organ transplantation and disease associations. The method has a relatively good ability to distinguish closely related

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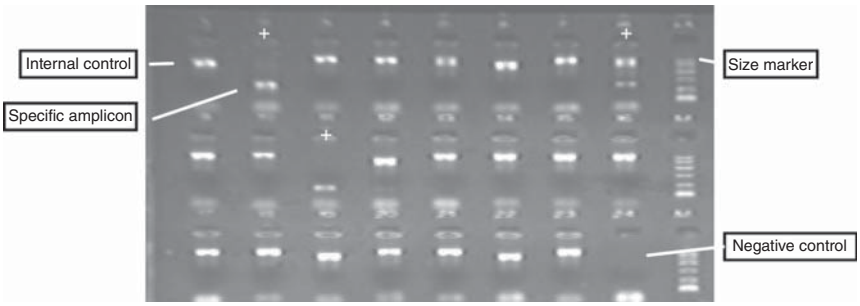

Figure 19.2 PCR-SSP gel electrophoresis. HLA typing is performed by a panel of HLA allele or group-specific primers. The PCR will take place only for primer mixes completely complementary to the target DNA. Each primer mix also contains internal positive control that amplifies a fragment with a length different from the HLA-specific amplicon. A size marker is used in gel electrophoresis to define the size of specific amplicons and internal controls.

allele combinations. The main restrictions of PCR-SSP are the low throughput due to the use of a large number of primer mixes, the relatively high amount of DNA needed, and the manual final step of the assay – agarose gel electrophoresis.

Although the classical PCR-SSP methods have been used routinely by H&I laboratories, in the last decade real-time PCR-SSP methods with increased sensitivity and less time for typing have been used as no post-amplification steps are needed. Two main types of real-time PCR-SSP methods, TaqMan assay and HLA melt curve analyses, have been developed. In the real-time TaqMan PCR-SSP, each allele- or group-specific primer mix also includes at least one non-extendable in the extension phase of the PCR TaqMan probe, designed to bind to sequence between the primers' binding sites. The TaqMan probes are labeled with 2 markers – fluorescent reporter dye at the 5′ end and a quenching marker at the 3′ end. The fluorescent reporter dye is excited with light at specific wavelength and emits light at different wavelengths. When the probe is intact, the fluorescence energy response occurs, and the emission energy from the reporter dye is absorbed by the quencher, and no or little emission can be detected [47]. If the specific primers are complementary to the target DNA, the PCR will start. In the annealing phase, the primers and probes will bind to the target sequences, and the elongation will take place. The method uses the 5′ nuclease activity of Taq polymerase [48], and when the Taq polymerase reaches the probe, it will degrade it. The hydrolysis of the probe releases the quenching of the reporter's fluorescent emission. At the end of each extension step of the PCR, the light emitted from the free reporter dye is detected by the digital camera of the real-time PCR device. Within each PCR cycle, the amount of free reporter dye increases proportionally to the amount of the PCR product. For each filter combination, a graph showing the change of fluorescence against the cycle number is plotted (Figure 19.3). The real-time PCR devices allow multiple reporter dyes to be used in each PCR well, and a combination of different independent sets of PCR-SSP primers and probes with different reporter dyes allows several independent reactions to take place in a single well. The combination of the primer set and probe allows for

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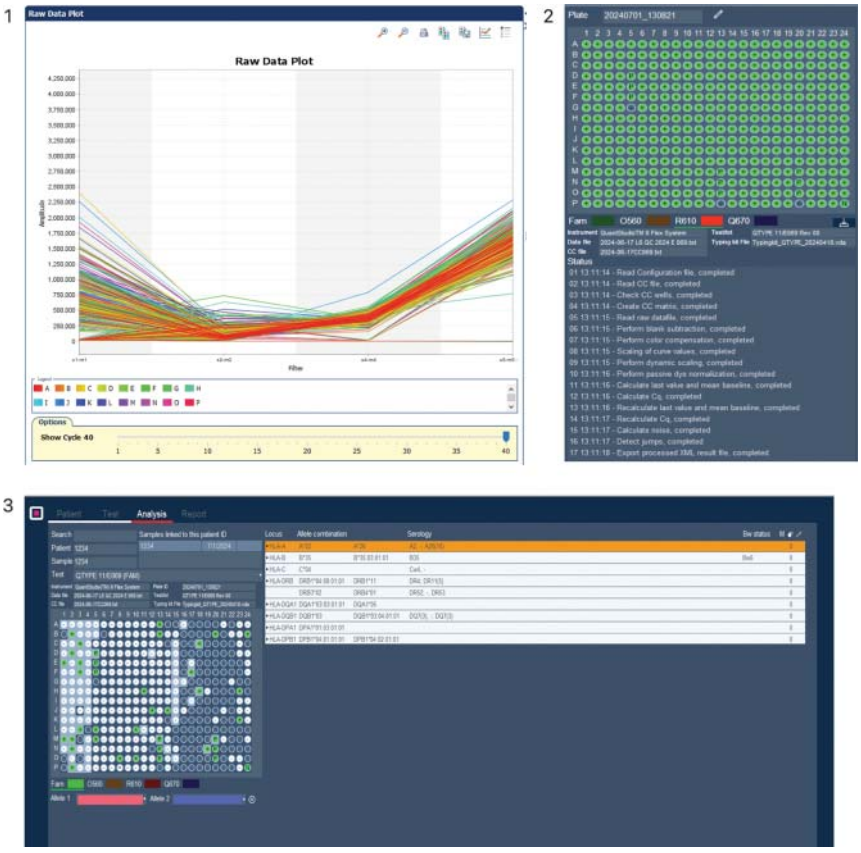

Figure 19.3 Real-time PCR-SSP TaqMan assay. HLA typing is performed by a panel of HLA allele or group-specific primers. Each mix also includes at least one non-extendable in the extension phase of the PCR TaqMan probe, designed to bind to sequence between the primers' binding sites and labeled with two markers – fluorescent reporter dye at the 5′ end and a quenching marker at the 3′ end. When the probe is intact, the fluorescence energy response occurs, the emission energy from the reporter dye is absorbed by the quencher, and no or little emission can be detected. If specific PCR starts, the Taq polymerase will degrade the probe, and the light emitted from the free reporter dye is detected. The real-time PCR devices allow multiple reporter dyes to be used in each PCR well. 1) A graph showing the change of fluorescence against the cycle number for each filter combination. 2) and 3) Score 6 analysis of QTYPE 11 (GareDx, USA) – the kits utilize two functions of the amplification curve to determine a positive result for each reaction: the Cq value and the overall fluorescent value during the final cycle. Internal amplification control is included in each well. The values from the HLA assays are normalized to the values from the internal controls.

an increase in the resolution of the assay. Modifications of the method with minor groove binding (MGB) probes with increased melting temperature allowed the use of shorter probes [49]. Initially, PCR-SSP TaqMan assays were applied for typing of single specificities such as HLA-DQB1*06:02 [50], HLA-DRB1*04 subtypes [51], HLA-B*57:01 [49], or single loci (HLA-DR) [52, 53]. Commercially available kits (such as QTYPE 11, CareDx, United States) using this technology allow the analysis

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of 11 loci with intermediate resolution in 384 formats. The kits utilize two functions of the amplification curve to determine a positive result for each reaction: the Cq value and the overall fluorescent value during the final cycle. Internal amplification control, amplifying the conserved human growth hormone gene, is included in each well. The values from the HLA assays are normalized to the values from the internal controls allowing to compensate for any variation in DNA amount or pipetting errors. Additionally, each plate includes positive control wells for each fluorescent channel and a negative control with no DNA to monitor possible contamination. Another type of real-time PCR-SSP method utilizes melting curve analysis. Similar to the classical PCR-SSP method, each well of the plate contains a specific HLA allele or allele group primer mix and an internal control primer pair. A fluorescent dye (SYBR Green) is included in the master mix. Melt curve technology is used to detect amplicons produced during PCR. The dye fluoresces when it is bound to the amplicon, and no fluorescence is detected when the dye is free in the solution. A melt curve is produced at the temperature change from 65 °C when double-strand amplicons are present to 95 °C when amplicons are desaturated and SYBR Green is released in the solution. Specific amplicons will melt at the same temperature allowing to detect specific HLA alleles or allele groups. The internal control melts at a lower temperature than the specific amplicon and is absent in the presence of the specific HLA allele or group of alleles. PCR-SSP melt curve analysis methods have been developed for typing of single alleles (HLA-B*57:01, DQ2/8) [54, 55] or loci (HLA-DR) [56]. Commercial kits based on this technology allowing intermediate typing of 11 HLA loci in 384 well format are available (LinkSeq HLA typing kits, Thermo Fisher Scientific, USA). Both real-time PCR-SSP methods are fast and allow HLA typing to be completed in less than one hour. Considering the higher number of loci typed (HLA-A, HLA-B, HLA-C-DRB1, HLA-DRB3/4/5, HLA-DQA1, HLA-DQB1, HLA-DPB1, and HLA-DPA1) and the higher level of resolution compared to conventional PCR-SSP and no manual steps once the PCR plate is sealed and placed in the machine, many laboratories apply these methods for typing of deceased donors. Current studies showed that the antigen level of resolution of commercially available kits makes them suitable for typing of donors and sensitized patients in order to predict unacceptable mismatches in virtual crossmatch and to select donors for sensitized patients [57]. However, these test have some limitations, including the requirements for relatively high amount of high quality DNA; lack of full sequence information for all HLA alleles, particularly outside exons 2 and 3 for HLA class I and exon 2 for HLA class II and introns; and therefore, some of the multiple primer mixes or probes may not be completely matched with the target sequence. Due to the high similarity between genes in the HLA complex, some of the primer mixes may amplify other genes, including pseudogenes; the commercially available kits have high cost and require experienced technicians, use and maintenance of real-time PCR machines.

Despite the fact that many clinical protocols for solid organ transplantation will require a transition to high-resolution typing, low-resolution HLA typing methods still have clinical applications. PCR-SSP and PCR-SSOP methods are well standardized and allow reliable typing at low resolution and intermediate resolution.

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20

High-Resolution HLA Typing

Sanger Sequencing, NGS, Long-range Sequencing

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20.1 Introduction

In stem cell transplantation, clinicians try to select the best matching donor for their patient, among other important factors, largely based on the human leukocyte antigen (HLA) genotype of the patient and potential donor. Since the immune system of the donor is transplanted to the patient, after a successful transplantation, the donor's immune cells are present in all parts of the patient's body. This also means that if graft versus host disease (GvHD) develops, this may involve a response of the whole body. HLA matching to the smallest detail of the HLA molecules reduces the chance of developing GvHD. HLA typing preferably is done by sequencing the relevant part of the DNA region encompassing the HLA genes, the so-called sequencing-based typing (SBT) method. SBT will determine the nucleotides of the alleles of both the donor and the recipient, which can be translated into the protein sequence. Developments in the sequencing technologies from Sanger-based sequencing to either short-read next-generation sequencing (NGS) or long-read NGS improved SBT significantly. However, the diversity of the HLA genes, which are among the most polymorphic gene families known, hampers even the sequencing method to uniquely identify the alleles of donor and recipient.

Also, for solid organ transplantation, accurate matching of HLA genes is important. But in situations where the organ is derived from a deceased donor, the time to complete HLA genotyping is rather restricted. This limits the option of choice for HLA typing to low-resolution or medium-resolution level methodologies like sequence-specific primers (SSP-PCR) or sequence-specific oligonucleotides probes (PCR-SSOP) by Luminex, hence the accuracy of HLA typing. Recent developments in rapid sequencing using nanopores now seem to make NGS applicable for HLA typing for solid organ transplantation.

In this chapter, the development of SBT is described. This includes sequencing technologies like Sanger sequencing and NGS, as well as the basics of data analysis, the HLA typing methods, possibilities, restrictions, and reliability of the obtained results. At the end of this chapter, the richness of the NGS data is illustrated with some special cases in which the data show insight into the patient's situation.

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20.2 HLA Genes

High-resolution genotyping of the HLA genes can be achieved by sequencing-based typing (SBT) strategies. Through time, the actual genes to be sequenced have changed, as well as what the relevant regions are that should be determined. Since the peptide-binding region is considered the most immunogenic region, the earlier protocols were restricted to sequencing exon two for the Class II HLA genes, and exons two and three for the Class I HLA genes. The identification of many coding polymorphisms outside these regions resulted in changing protocol to include more exons. Including more regions resolved ambiguous results, and in addition, it enabled research on determining the nature and the importance of these polymorphisms.

Currently, whole-gene polymerase chain reaction (PCR) amplifications are the basis for NGS-SBT, which also enables studying the influence of non-coding regions. Technical restrictions still prevent routinely including all regions for all genes. For instance, class II HLA genes have very large intron one regions, which in some genes require PCR amplification of a gene in two separate fragments: part of intron one is then not included in the PCR product and cannot be sequenced, hence determined. The development of gene capture methods may overcome this restriction, since these methods do not involve a primary PCR, specific for the gene of interest.

Currently, most typing commercial and in-house-developed protocols do include all classical HLA genes, HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DRB3, HLA-DRB4, HLA-DRB5, HLA-DQB1, HLA-DQA1, HLA-DPB1, and HLA-DPA1. However, HLA-DRA1 is not routinely included.

SBT by NGS allows adding additional genes, including non-HLA genes, to the protocol without compromising the protocols for analysis. However, adding more genes or adding additional regions influences the number of samples that can be managed in a single workflow.

20.3 PCR Amplification

Most DNA techniques described in this chapter use PCR amplification after the isolation of genomic DNA. The quality of the PCR products also determines the quality of the typing results. For Sanger sequencing, it is required that for each single gene, an amplification by PCR is carried out separately. One of the powerful features of NGS is that the DNA sequence of each individual DNA molecule from a PCR product is determined. Bioinformatics methods will sort and combine these results with the specific gene. This also allows the pooling of PCR products derived from either different genes or even different samples. Several multiplex PCR products are nowadays available. The software packages for analysis and their bioinformatics algorithms can sort the reads to the relevant genes and samples.

For high-resolution HLA typing, full coverage of a gene is preferred. This means that the whole gene is amplified, sequenced, and analyzed. Figure 20.1 shows the

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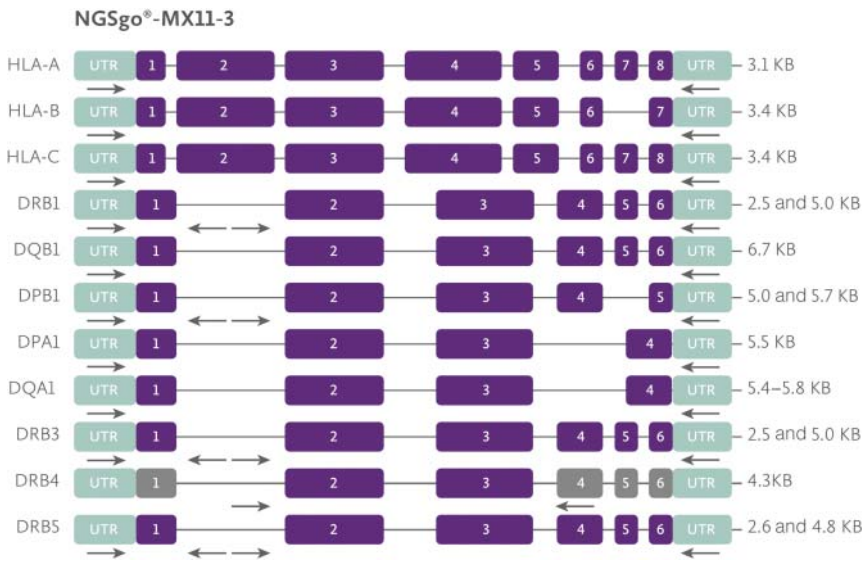

Figure 20.1 Amplification strategy for 11 HLA loci. For the Class I genes, HLA-DQB1, HLA-DQA1, and HLA-DPA1, the amplification includes the whole gene. For the other genes, the amplification is split into two regions due to the long length of intron 1. HLA-DRB4 is only partly covered.

gene coverage of the NGSgo®-MX11-3 approach of GenDx[1] in which 11 HLA loci are amplified. Except for HLA-DRB4, the full coding regions are covered.

HLA genes and pseudogenes show high homology, which may in some DNA samples or amplification protocols result in co-amplification of genes that were not targeted. An example of high homology between HLA-A and pseudogene HLA-Y is shown in Figure 20.2, showing that they differ by only 4% or 5% of the nucleotides. Other examples are within the HLA-DRB gene family showing high homology encompassing both the expressed genes and pseudogenes. Figure 20.3 shows an example of identical exon 2 sequences of alleles of two different genes. Bioinformatic methods have been developed to distinguish the DNA sequence derived from these co-amplified regions.

Crucial for the reliability of a PCR-based HLA typing method is that the PCR will amplify all alleles. However, allelic dropout, in which one allele is not amplified, or not as efficiently amplified, although rare in the current products, can never be excluded. This may result in false-homozygous results. Therefore for homozygous typing results, additional bioinformatic tools should be applied or laboratory tests should be carried out.

The PCR products are sequenced and analyzed to an HLA typing result.

1 NGSgo®-MX11-3, GenDx, Utrecht, the Netherlands. www.GenDx.com.

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```
A*26:01 ATGGCCGTCA TGGCGCCCCG AACCCTCGTC CTGCTACTCT CGGGGGCCCT GGCCCTGACC
Y*01:01 -----G---G ---------- -------C-- ---------- ---------- ----------

A*26:01 CAGACCTGGG CGG | GCTCCCA CTCCATGAGG TATTTCTACA CCTCCGTGTC CCGGCCCGGC
Y*01:01 ---------- --- | ------- ---------- -------C-- -A-------- ----------

A*26:01 CGCGGGGAGC CCCGCTTCAT CGCCGTGGGC TACGTGGACG ACACGCAGTT CGTGCGGTTC
Y*01:01 A-T--A---- ---------- ---A------ ---------- ---------- ----------

A*26:01 GACAGCGACG CCGCGAGCCA GAGGATGGAG CCGCGGGCGC CGTGGATAGA GCAGGAGGGG
Y*01:01 ---------- ---------- ---------- ---------- -------G-- --------A-

A*26:01 CCGGAGTATT GGGACCGGAA CACACGGAAT GTGAAGGCCC ACTCACAGAC TGACCGAGCG
Y*01:01 ---------- --------C- G----A--TC TCC---A--A --G------T -----T--A-

A*26:01 AACCTGGGGA CCCTGCGCGG CTACTACAAC CAGAGCGAGG ACG | GTTCTCA CACCATCCAG
Y*01:01 -G----C--- T-GC--T-C- ---------- ---------- C-- | ------- ----------

A*26:01 AGGATGTATG GCTGCGACGT GGGGCCGGAC GGGCGCTTCC TCCGCGGGTA CCAGCAGGAC
Y*01:01 -------C-- ---------- ----T----- ---------- ---------- --G-------

A*26:01 GCTTACGACG GCAAGGATTA CATCGCCCTG AACGAGGACC TGCGCTCTTG GACCGCGGCG
Y*01:01 --C------- ---------- ---------- ---------- ---------- ----------

A*26:01 GACATGGCGG CTCAGATCAC CCAGCGCAAG TGGGAGACGG CCCATGAGGC GGAGCAGTGG
Y*01:01 ---------- ---------- ---------- ------G--- ---G-C---- --------T-

A*26:01 AGAGCCTACC TGGAGGGCCG GTGCGTGGAG TGGCTCCGCA GATACCTGGA GAACGGGAAG
Y*01:01 ---------- --------GA ----A----- ---------- ---------- ----------

A*26:01 GAGACGCTGC AGCGCACGG | A CGCCCCCAAG ACGCATATGA CTCACCACGC TGTCTCTGAC
Y*01:01 ---------- --------- | - ---------- ---------- ---------- ----------

A*26:01 CATGAGGCCA CCCTGAGGTG CTGGGCCCTG AGCTTCTACC CTGCGGAGAT CACACTGACC
Y*01:01 A--------- ---------- ---A------ ---------- ---------- ----------

A*26:01 TGGCAGCGGG ATGGGGAGGA CCAGACCCAG GACACGGAGC TCGTGGAGAC CAGGCCTGCA
Y*01:01 ---------- ---------- ---------- ---------- ---------- ----------

A*26:01 GGGGATGGGA CCTTCCAGAA GTGGGCGTCT GTGGTGGTGC CTTCTGGACA GGAGCAGAGA
Y*01:01 --------A- T--------- -------G-- ---------- --------G- ----------

A*26:01 TACACCTGCC ATGTGCAGCA TGAGGGTCTG CCCAAGCCCC TCACCCTGAG ATGGG | AGCCG
Y*01:01 ---------- ---------- ---------- ---------- ---------- ----- | -----

A*26:01 TCTTCCCAGC CCACCATCCC CATCGTGGGC ATCATTGCTG GCCTGGTTCT CTTTGGAGCT
Y*01:01 --------T- ---------- ---------- ---C------ ---------- ----------

A*26:01 GTGATCGCTG GAGCTGTGGT CGCTGCTGTG ATGTGGAGGA GGAAGAGCTC AG | ATAGAAAA
Y*01:01 ---------- ---------- ---------- ---------- ---------- -- | --------

A*26:01 GGAGGGAGCT ACTCTCAGGC TGCAA | GCAGT GACAGTGCCC AGGGCTCTGA TATGTCTCTC
Y*01:01 ---------- ---------- ----- | ----- ----T----- ---------- -G--------

A*26:01 ACAGCTTGTA AAG | TGTGA |
Y*01:01 ---------- --- | ----- |
```

Figure 20.2 Alignment of the exons of HLA-A*26:01:01:01 and HLA-Y*01:01 showing the high homology between HLA-A and the pseudogene HLA-Y. Y*01:01 differs by 54 from the 1098 (5%) basepairs from A*26:01:01:01. Considering the whole gene shows that only 115 of the 2920 (4%) basepairs differ between the HLA-A allele and HLA-Y allele (alignment not shown).

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```
AA Codon                        10                  15                  20                  25
DRB1*14:141       CA CGT TTC TTG GAG CTG CTT AAG TCT GAG TGT CAT TTC TTC AAT GGG ACG GAG CGG GTG CGG TTC CTG GAG AGA
DRB3*02:02:01:01   -- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

AA Codon            30                  35                  40                  45                  50
DRB1*14:141       CAC TTC CAT AAC CAG GAG GAG TAC GCG CGC TTC GAC AGC GAC GTG GGG GAG TAC CGG GCG GTG AGG GAG CTG GGG
DRB3*02:02:01:01  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

AA Codon            55                  60                  65                  70                  75
DRB1*14:141       CGG CCT GAT GCC GAG TAC TGG AAC AGC CAG AAG GAC CTC CTG GAG CAG AAG CGG GGC CAG GTG GAC AAT TAC TGC
DRB3*02:02:01:01  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

AA Codon            80                  85                  90
DRB1*14:141       AGA CAC AAC TAC GGG GTT GGT GAG AGC TTC ACA GTG CAG CGG CGA G
DRB3*02:02:01:01  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -
```

Figure 20.3 Example of homology between two HLA genes. Identical exon 2 sequences of DRB1*14:141 and DRB3*02:02:01:01.

20.4 Gene Capture Method

Recently, DNA capture methods are applied in which the HLA genes are enriched from genomic DNA by capture techniques. An example is AlloSeq Tx from CareDx[2]. This approach involves fragmentation of genomic DNA followed by ligation of index adapters and PCR amplification to increase the amount of DNA. Genes of interest are captured with biotinylated probes and enriched for sequencing on an Illumina system. This method does not have the limitations of target-specific PCR amplification and may cover whole genes without limitation. Other genes are easy to add. However, a higher sequencing capacity is required since the regions covered are larger compared to PCR-based methods, such as the complete intron 1 of class II genes and regions beyond the untranslated region (UTRs).

20.5 SBT by Sanger Sequencing

Sanger sequencing was developed in 1977 [1], but was commercialized by Applied Biosystems in 1986. After the amplification of a gene by PCR, a sequencing reaction is performed. The starting material for sequencing is a single-stranded DNA template obtained from the primary PCR. A sequencing primer, complementary to the template, is, after annealing, subjected to another PCR. The sequencing reaction mix contains a mix of normal nucleotides deoxynucleotide triphosphate (dNTPs) and chain-terminating dideoxynucleotide triphosphate (ddNTPs), which are fluorescently labeled. In the reaction, randomly the complementary dNTP or ddNTP is incorporated. When a ddNTP is incorporated, the fragment can no longer be extended, but becomes labeled with the fluorescent dye of that ddNTP. Using electrophoresis, the fragments are separated based on length. All fragments with the same length and ending with the same nucleotide are measured by exciting the fluorescently labeled fragments. Each nucleotide, A, C, G, and T, has a different fluorescent color. The length distribution and the color of the fragments are measured as an electropherogram which is converted into a DNA sequence. Examples of electropherograms and the base calls are depicted in Figure 20.4.

The design of the sequencing primer is crucial for the assay, allowing highly specific binding. Already a few nucleotides downstream of the sequencing primer, the

2 AlloSeq Tx, CareDx, Brisbane Ca, USA. Caredx.com.

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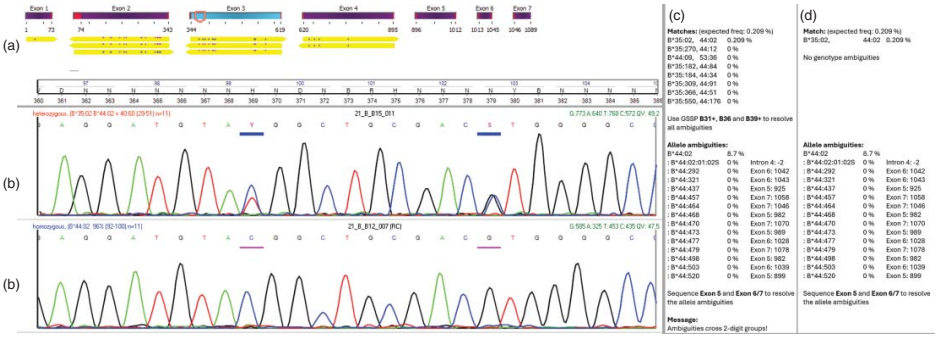

Figure 20.4 A screenshot of an HLA-B typing using SBT by Sanger sequencing. (a): A representation of the exons of HLA-B; nine yellow arrows represent nine aligned traces. The arrow indicates the alignment orientation. The dots are at the heterozygous positions of the sample. Blue dots in a heterozygous sequencing read. Pink dots in a homozygous read obtained by using a group-specific sequencing primers (GSSP). (b): Parts of two electropherograms are shown. The peaks are color coded for nucleotide content. On top of each electropherogram, the assigned nucleotides are shown. Notice the Y=CT and S=GC encoding heterozygous basecalls. (c): The typing result if no GSSPs were applied. In this analysis, a total of 1,130 genotypes match with the data, using the IPD-IMGT/HLA 3.54. In the report, the lists are reduced to an optimized form leaving out any ambiguities that only involve the third or fourth field. Eight genotype ambiguities remain, and 14 different coding variants of B*44:02 are not discriminated. All B*44 allele ambiguities could have been resolved by including the sequences of exons 5, 6, and 7. All genotype ambiguities could have been resolved by applying 3 GSSP reactions. (d): The results of the same sample but after applying the GSSPs in exons 2 and 3, which resolved all eight genotype ambiguities excluding the presence of 8 genotypes all with rare alleles.

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sequence can be determined, typically over a distance of about 300–400 nucleotides. To perform an HLA class I genotyping, at least the sequence of the core regions should be sequenced, but in most cases extra reactions are necessary to cover relevant polymorphisms.

Three issues caused Sanger SBT to become less suitable for routine HLA typing in stem cell transplantation:

First, electrophoresis results show artifacts that sometimes are difficult to interpret. To resolve this issue, it is required to sequence each region in forward and reverse orientations. For sequencing, e.g. HLA-A, the exons 2, 3, and 4, six sequencing reactions are necessary. Figure 20.4 is a screenshot of the software package SBTengine®, GenDx[3] [2], the Netherlands. In this screenshot, 9 electropherograms including exon 1 and 2 electropherograms of allele-specific sequencing reactions, their alignment with HLA-B, and the HLA typing results are shown.

Second, many of the HLA alleles share motifs. Sanger sequencing produces a mixed sequence of the two alleles. At positions where the two alleles differ, both nucleotides are represented as a peak. However, the cis or trans relation to nucleotides at other heterozygous positions cannot be determined. As a consequence, these basic sequences frequently match with several genotypes, called ambiguities. Further in this chapter, more details about ambiguities are described. Ambiguities can be resolved by additional sequencing reactions that are specific for one of the two alleles present, allowing determination of the cis or trans relation of the nucleotides, thus resolving the ambiguous result. The specific sequencing primers are called GSSPs [3], motif-specific primers (MSPs), heterozygous ambiguity resolving primers (HARPs) [4], or other company-specific names. Figure 20.4 illustrates how GSSPs can resolve ambiguous typing results.

Third, the number of identified HLA alleles continuously increases, since the start of NGS even dramatically. Also, many coding differences are recognized outside the core regions. This leads to needing more basic sequencing reactions covering more exons, and the need to increase the number of GSSPs. Even with the most optimal set of GSSPs, ambiguities will remain at a level that is not considered acceptable anymore.

20.6 HLA Typing by NGS SBT

Next-generation sequencing (NGS), also known as massively parallel sequencing, is a powerful sequencing technique that enables obtaining nucleotide sequences of individual DNA molecules. These DNA sequences are called reads. The technique is extremely useful for high-resolution typing. Initially, both equipment and protocols were complex and expensive, and only affordable by larger research institutions sequencing large numbers of samples. The development of smaller sequencing equipment, suitable for relatively low-throughput, typical for an HLA typing laboratory, and easier to apply sequencing protocols, made NGS efficient for routine HLA typing.

3 SBTengine®, GenDx, Utrecht, the Netherlands. www.GenDx.com. This software package is designed by one of the authors, and further developed and used since 2005.

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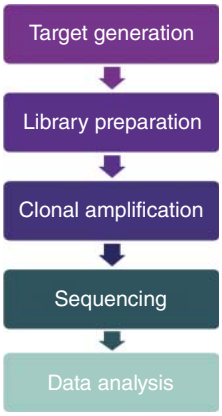

Figure 20.5 The basic steps to perform SBT by NGS.

NGS sequencers generate files with reads. Each read is the nucleotide sequence of a single DNA molecule and is the basis for the analysis and the typing result.

Figure 20.5 shows the general steps for HLA typing by SBT:

Target generation is the PCR amplification of either the gene(s) of interest or the captured DNA fragments including the genes of interest.

Library preparation concerns the preparation of the DNA for sequencing. The exact protocol depends on the sequencing method used but may involve fragmentation of the DNA if a short-read sequencer is used and involves ligating adapter and indices to the fragments necessary for the sequencing process and determining to which sample a particular read belongs.

The NGS-based SBT methods show the next developments:

- **Roche 454 NGS sequencer**: This was the first sequencer used for which HLA typing protocols were developed [5]. Since this method is not applied anymore, it will not be described in detail.
- **Short-read sequencing**: This equipment was developed to produce reads with a typical length of 300–1,000 nucleotides. The MiSeq, ISeq, and miniSeq produced by Illumina Inc.[4] and the IonTorrent instruments produced by Thermo Fisher Scientific Inc.[5] are the platforms mostly used. These instruments produce high-quality data and are widely used for high-resolution HLA typing.
- **Long-read sequencing**: The development of NGS sequencers that can determine the sequence of much longer DNA molecules enabled the capacity to obtain individual sequences covering whole genes. The two most used systems are the PacBio Sequel II[e6] and the Oxford Nanopore Technologies (ONT)[7] MinIon systems. These systems succeed in measuring single DNA molecules. Recently, the technical and bioinformatics development resulted in improving the data quality extensively.

4 Illumina Inc, San Diego, CA, USA. www.illumina.com
5 Thermo Fisher Scientific Inc. Waltham, MA, USA. www.thermofisher.com
6 Pacific Bioscience Inc. Menlo Park, CA USA. www.pacb.com.
7 Oxford Nanopore Technologies. Oxford, UK. nanoporetech.com

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The ONT equipment and protocols development also makes it possible to obtain high-resolution HLA typing results from DNA to typing within a couple of hours.

The analysis of the data from the sequences of the reads to the HLA typing results is crucial and will be explained in each section.

20.7 HLA Typing by Short-read Sequencing

Determining the DNA sequence of the whole gene enables high-resolution HLA typing results. However, the HLA genes are larger than the lengths of the reads obtained by short-read NGS sequencers. Therefore, after PCR amplification of the genes, the PCR product must be fragmented. Each HLA typing approach has its own specific fragmentation chemistry and protocols, mostly based on enzymatic reactions or by sonication. For the actual protocols, visit the kit providers.

The fragmentation process will generate random fragments within a specific size range (See Figure 20.6). Adapters are ligated to the fragment. The general adapters have specific sequences necessary for the actual sequencing reactions. In addition, indices sequences are added which are sample specific, and will be included in the final DNA sequence. All reads originating from a particular sample can be selected based on identity of the indices sequences. The exact configuration and protocol differ per sequencing platform.

In short-read sequencing protocols, individual-labeled DNA molecules generate fluorescent signals that are too weak to be determined. Therefore, the DNA fragments with adapters and indices have to be amplified again in isolation, the clonal amplification, before a short read sequencer is able to detect the signal.

Two mostly used short-read sequencing systems are given as follows (see Figure 20.7).

- Illumina MiSeq and compatible platforms (www.illumina.com) (see Figure 20.7a)
  - Adding adaptors to the DNA fragments.
  - Annealing to primers fixed on a solid-state sequencing chip or flow cell.
  - Clonal amplification by bridge amplification generates spatially separated clusters of identical DNA fragments.
  - Sequencing is based on a sequencing-by-synthesis (SBS) method. In cycles, fluorescent-labeled dNTPs, modified to prevent further extension, are added resulting in each cluster fluorescing in the color of the nucleotide incorporated. After measuring the fluorescence, the fluorophore is chemically quenched, and the extension blocking is removed, and the next cycle is initiated.

Figure 20.6 Short reads overlapping throughout the amplicon. Reads of two different alleles have been sequenced (gray vs. purple).

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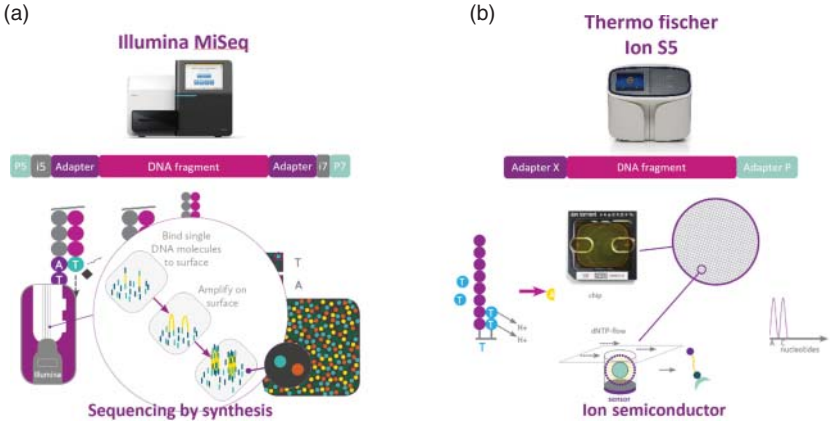

Figure 20.7 The most common short-read sequencers. (a): Illumina MiSeq instrument. To each DNA fragment, an adapter is ligated. i5 and i7 indices are the barcodes used to determine to which sample the fragment belongs to. P5 and P7 are binding regions used for binding to the flow cell surface. (b): Thermo Fisher Ion S5 instrument. Source: Adapter X and Adapter P. The P adapter will bind to the ISP beads for the clonal amplification. Adapter X contains the barcode used to determine to which sample the fragment belongs.

  - The fluorescence signals are converted into a DNA sequence.
  - Paired-end sequencing can be performed in which a DNA fragment is measured from both sides. The advantages will be described in the data analysis section of this chapter.
- Thermo Fisher Ion Torrent and compatible platforms (www.thermofisher.com) (see Figure 20.7b)
  - Adding adaptor to the DNA fragments.
  - Clonal amplification is performed in an emulsion PCR.
    - Binding of single fragments to beads.
    - The beads are covered with DNA complementary to the adapters.
    - The clonal amplification is accomplished by emulsion PCR.
  - The beads are added to a chip with small holes which can occupy a single bead.
  - In cycles, in consecutive rounds, either A, C, T, or G nucleotide as dNTP is added.
    - Once bound, a hydrogen ion is released which is measured.
    - The pH change is proportional to the number of actual dNTPs bound.
  - Signals are converted into a DNA sequence.

The two mentioned brands are not the only companies that offer short-read sequencers. The two alternatives are given as follows:

- **MGI DNBSEQ sequencers (**www.mgi-tech.eu**)**: This sequencer uses DNA Nanoballs (DNB) for signal amplification. DNBs are generated using rolling circle replication (RCR). After PCR amplification of the target genes, and fragmentation, the fragments are circularized. RCR employs a high-fidelity DNA polymerase, ensuring that each amplification cycle uses the original DNA circle

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as the template. Each DNB contains about 300–500 copies of the original DNA fragment. The DNBs are loaded to a patterned array and sequenced generated paired-end reads.

- **AVITI sequencers of element biosciences (**www.elementbiosciences.com**)**: This sequencer utilized rolling circle amplification (RCA) as a method for signal amplification. The sequencer is able to generate paired-end sequences of 2×300 bp.

20.7.1 Paired-end Sequencing

Illumina short-read sequencers can apply paired-end sequencing. During library preparation, adapters are added to DNA fragments such that on one end P5 is attached and on the other end P7 is attached. This allows proper amplification on the Illumina chip. Subsequently, the sequencer will first determine 150 bp from one end and then 150 bp from the other end. For each end, a fastq file is generated. The names of the files will be identical except that the first will have _R1_ in the filename, and the second will have_R2_ in the filename. This allows that during analysis, the reads from each DNA fragment can be paired.

Paired-end increases phasing possibilities

Consider reads of length 300 nucleotides. If the distance between two heterozygous positions is 301 nucleotides or more, not a single read will cover both positions, and it will not be possible to phase the nucleotides. If paired-end reads of 2×150 nucleotides are generated for the same sample, there have been DNA fragments longer than 300 bp of which both sides are sequenced (see Figure 20.8). After pairing the two sequences, the nucleotide content at the heterozygous positions can be imputed.

The data quality of short-read methods is high. However, the limited length of the individual reads, which are randomly distributed over the amplified genes, stresses the following data analysis. Some reads may not be uniquely assigned to the HLA gene due to the homology between the genes. In addition, if heterozygous positions are separated by a large region, short reads may not enable the phasing between those, which may result in ambiguous results. In practice, these cases are rare.

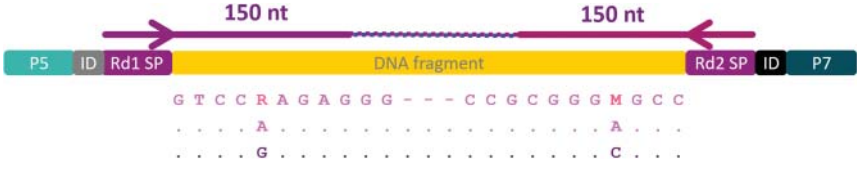

Figure 20.8 A fragment with adapter and indices. The original DNA fragment is indicated in yellow, and in this example, it is longer than 300 bp. From each end, 150 bp are sequenced. After aligning, although in the middle a part is not sequenced, the combined sequence covers the two heterozygous positions. This paired read can now be used to impute the actual nucleotides at the two positions within one allele without ambiguity.

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20.8 HLA Typing by Long-read Sequencing

The development of long-read sequencing, or third-generation sequencing, enabled new ways of analyzing genomic data. The length of individual reads can be 10,000 base pairs long. Even lengths of almost 1,000,000 bases are reported. This opened new ways in determining larger structural variants and unraveling even complex genetic regions. This, however, comes at the cost of low-quality data at individual base position. Recently, technology and bioinformatic analysis also have been optimized to increase data quality. This limits the typical lengths of individual reads but enables the use of long-read sequencing for HLA typing. The length of reads still is longer than the individual HLA genes. Application for HLA typing will generate reads covering the whole region amplified (see Figure 20.9).

Two companies are pioneers of long-read sequencing (see Figure 20.10). Both succeed in real-time measuring of single DNA molecules, without the need for clonal amplification.

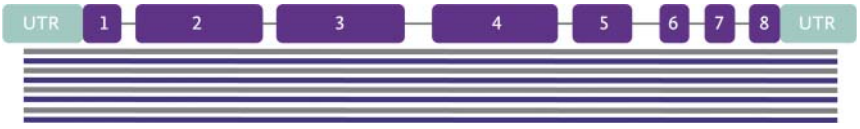

Figure 20.9 Reads of two different alleles have been sequenced (grey vs. purple) by long read sequencing. Individual reads cover the whole gene.

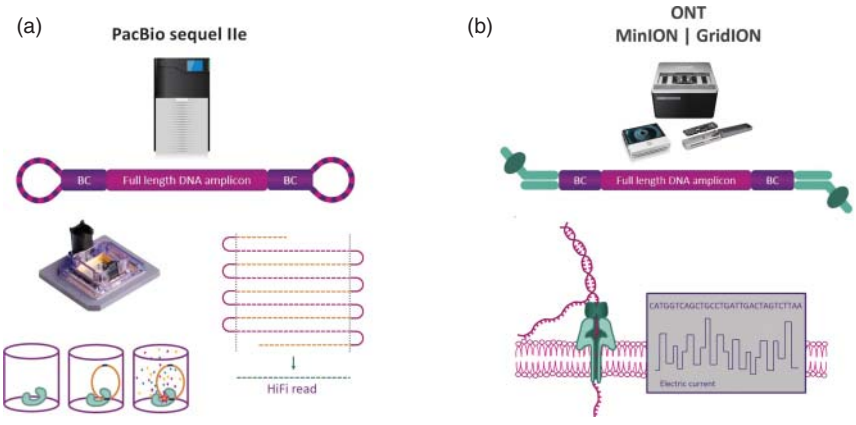

Figure 20.10 The most common long-read sequencers. (a): The PacBio Sequel IIe instrument. PCR fragments are circularized and called a SMRTbell. By sequencing the SMRTbell multiple times around, HiFi reads are generated which are of high quality. (b): The MinIon and GridIon instruments. DNA molecules are guided by a motor protein through a nanopore. The DNA sequence is determined by analyzing the change in electrical current.

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20.8.1 Pacific Biosciences (PacBio)

PacBio developed single-molecule real-time (SMRT) sequencing. Single-molecule imaging of molecular interactions uses zero-mode waveguides (ZMWs). ZMWs are nanoapertures (typically ~100 nm diameter) in a thin metal film deposited on a glass substrate, each with a polymerase fixed at the bottom. When light enters the ZMWs from the bottom, a tightly confined evanescent field (20–30 nm thickness) is generated inside the ZMWs, enabling single-molecule fluorescence imaging in the presence of several μM of fluorophores in a solution. The introduction of high fidelity (HiFi) reads dramatically increased the quality of the reads (see Figure 20.10a). HiFi reads are generated as a consensus sequence from multiple passes around a circular molecule [6].

Among the PacBio equipment is the Sequel II, which produces HiFi reads. This equipment can be used for HLA typing. In addition, it is useful in unraveling more complex regions like the KIR genetic region. The Sequel II is developed for high-throughput sequencing.

20.8.2 Oxford Nanopore Technology (ONT)

ONT sequencing method uses a flow cell that contains nanopores, which are tiny holes, in an electro-resistant membrane. Guided by a motor protein, DNA molecules will pass through a nanopore (see Figure 20.10b). The electrical current through the nanopore will be changed by the DNA molecules. The change is dependent on the actual stretch of nucleotides in the pore. Typically, the pore occupies about a stretch of 6 bases. Analysis of these changes is decoded by basecalling algorithms resulting in a DNA sequence in real time.

The equipment of ONT is very small and continuously in development. Mostly used is the MinION sequencing device. The speed of the data depends on the computer hardware. Details are described on the ONT website. Recently, the quality of the reads has increased. Currently, ONT sequencing can also be applied routinely for HLA typing.

20.9 Ambiguities

Since the introduction of DNA technology for HLA typing, ambiguous results are encountered, which means that multiple genotypes match with the high-quality DNA data. This especially is cumbersome when the genotypes represent different serological groups. With the recognition of more and more alleles, the list of ambiguities for a locus typed by SBT by Sanger increases to a level that cannot be resolved anymore. SBT by NGS, when sequencing the whole gene, reduces the number of expected ambiguities dramatically.

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HLA typing based on SBT may result in two kinds of ambiguities:

(1) **Allele ambiguities**: These are caused when alleles differ in regions that are not included in the analysis. This can be caused by position(s), where alleles differ, are excluded for technical reasons, e.g. because they show amplification artifacts, or are difficult to sequence or align. Another cause is that the alleles differ outside the regions amplified by PCR, which becomes a moving target since more extended sequences of the UTRs of the genes become available. Alleles that only differ at these positions are considered 4th-field variants,

(2) **Genotype ambiguities or phase ambiguity**: A genotype ambiguity represents two genotypes of which all alleles have different sequences in the regions analyzed, but multiple allele combinations match perfectly with the sequence data. Figure 20.11 shows exon 1, 2, and 3 sequences of the four alleles of two genotypes, A*01:01:01:01,02:01:01:01 and A*02:36,36:04. All four alleles are different. If only the nucleotide content on each position was available, the two genotypes cannot be distinguished and would be reported as a genotype ambiguity. Phasing the nucleotides at the heterozygous positions in positions 560 with position 570 is essential to determine which two alleles determine the genotype in this sample. Phasing methodology is described in the next section.

In SBT by Sanger sequencing, exons 2 and 3 will not resolve these two genotypes unless extra reactions specific for individual alleles are applied. In general,

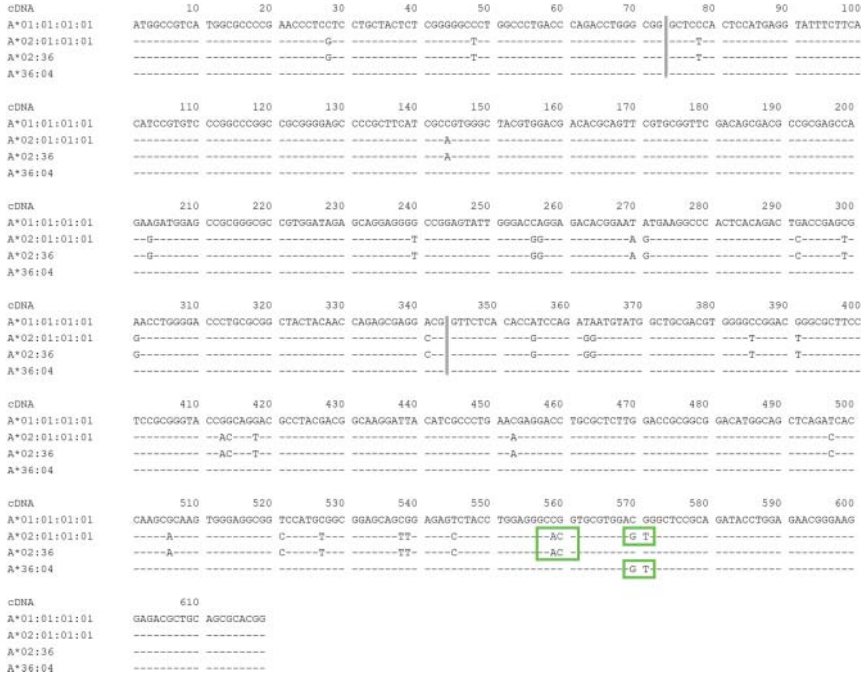

Figure 20.11 Sequences of the exons 1, 2, and 3 of the alleles of A*01:01,02:01 and A*02:36,36:04. Although all four alleles have different DNA sequences, phasing the nucleotides in positions 560 and 570 (green boxes) is essential to discriminate between the two genotypes.

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nowadays, the list of genotype ambiguities reported applying SBT by Sanger can be extremely long, and extra reactions are needed to resolve these ambiguities as much as possible.

SBT by NGS using short reads shows only a very low genotype ambiguity rate. Only when there is a large distance between the crucial heterozygous positions, or very low read depth, a genotype ambiguity can be expected. Examples are DPB1*04:01:01:01,04:02:01:02 and DPB1*105:01:01:002,126:01:01:01 (data not shown). Phasing needs to span a region of more than 4,300 nucleotides in length, which cannot be achieved by short-read sequencing.

SBT by NGS using long reads encompassing the whole gene allows phasing of virtually all heterozygote positions in all samples which thereby resolves almost all ambiguities.

20.10 Phasing

Phasing is an important step in the analysis process. Phasing is essential for high-resolution HLA typing. Phasing describes the bioinformatics method used to determine whether the two nucleotides are on the same (cis) or opposite (trans) chromosomes.

For a reliable analysis of the bases at a single position, a relatively low number of high-quality reads are sufficient. If, for instance, a position is covered by 100 reads, of which 40 have a G and 60 a C, then the conclusion is that the sample has an allele with G and a second allele with a C at that position. If the same data have at a position downstream 45 reads with a C at the second position, and 55 reads with a G, similarly, two alleles are present, one with a C and one with a G. Based on this information, however, Genotype 1 and Genotype 2 cannot be distinguished (see Figure 20.12), resulting in a genotype ambiguity. The example refers to Figure 20.11 used to illustrate genotype ambiguities.

In case a number of reads cover both positions, and they will either have GC or CG at these two positions, then this will confirm that Genotype 1 is present in the sample and not Genotype 2. In the HLA sample of Figure 20.11 used to illustrate genotype ambiguities, the counts between positions 560 and 570 are shown in Figure 20.13; 1384 reads cover both positions of which 623 support the GC nucleotide content

Position	560		570
Sample NGS data	G C	GTG…GGA	C G

Genotype 1	G	GTG…GGA	C	A*01:01:01:01
	C	GTG…GGA	G	A*02:01:01:01

Genotype 2	G	GTG…GGA	G	A*36:04
	C	GTG…GGA	C	A*02:36

Figure 20.12 Details of the nucleotide content of two genotypes. Positions 560 and 570 are heterozygous in the sample. The two genotypes share the nucleotides determined in the NGS data.

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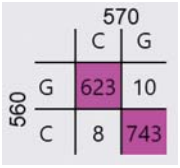

Figure 20.13 Phasing counts for positions 560 and 570. There are 623 reads with a G at position 560 and a C at position 570, and 743 reads with a C at position 560 and a G at position 570. Only 18 reads have other combinations of nucleotides at those positions. The two alleles have GC or CG at positions 560 and 570, and not CC or GG.

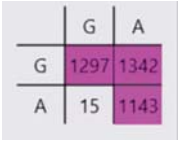

Figure 20.14 Phasing information between positions that do not describe a sample with only two alleles. Three patterns are identified: 1297 reads with GG, 1342 with GA, and 1143 with AG at these two positions.

of the A*01:01:01:01 allele of Genotype 1 and 743 support the CG content of the A*02:01:01:01 allele of Genotype 1. These 99% of all reads prove that this sample does not contain Genotype 2 but Genotype 1.

In a rare example of phasing data between two positions in Figure 20.14, three patterns are identified: GG, GA, and AG are each supported by approximately 1/3 of all reads. This indicates that the data do not represent a common diploid situation. This uncommon situation can be caused by:

- **Contamination**: The original PCR product was not pure.
- Multiple genes contribute to the data: A (pseudo)gene may have been amplified but not properly recognized during analysis.
- Chimeric situation, for instance, if the DNA sample is derived from a patient who had received a stem cell or solid organ transplantation, and the DNA source contains cells of the patient and donor.
- A mutation in a gene in part of the cells contributes to the PCR.

Phasing is illustrated for two positions. The actual phasing algorithms in NGS typing software extend these methods to analyze, whenever possible, more than two heterozygous positions. The patterns analysis covering multiple positions enables recognition of more subtle situations and improves statistical power.

The distance between the heterozygous positions on the same chromosome is important, since only reads covering both positions contribute to phasing: any read that ends or starts between the two positions cannot contribute to phasing. Two positions in proximity, as the positions 560 and 570 in the example will be covered by almost all reads covering the positions. However, if the read length is at a maximum of 150, and two heterozygous positions are about 140 basepairs apart, then only a small fraction of all reads covering one of the two heterozygous positions will cover both. This is a major reason why the read depth using short-read sequencing should be high. Long reads ideally will cover all heterozygous positions by each read, which reduces the need for high-read depth.

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20.11 Data Analysis

20.11.1 Sanger-based SBT Data Analysis

The sequencers generate data files with extension .ab1, which contain the readout of the electrophoresis, called a trace or electropherogram. Software is available for HLA typing using these files. The packages may differ in functionality, but all should be focusing on determining the matching genotypes and identification of artifacts or crucial positions. The screenshots in this chapter are generated using SBTengine®. Typically, for each exon to be analyzed, a forward reaction and a reversed reaction will be performed using two sequencing primers each at opposite sides of the exon, generating two traces. If the forward and reversed reactions have sequenced both alleles, the traces should give identical basecalls, if properly aligned. This will contribute to the identification and compensate for artifacts. Genotype ambiguities can be resolved by extra group-specific sequencing reactions.

Figure 20.4 shows a screenshot of an HLA-B typing, in which the exon 1 through exon 4 are sequenced, as can be recognized by the yellow arrows below the gene representation. In exon 2 and 3, two GSSP reactions are added. These reactions are specific for a specific allele. The peak patterns (see Figure 20.4b) show the electropherogram of a small part of exon 3. The top electropherogram represents both alleles, as can be recognized by the double peaks and the multibase codes $Y_{(weak)}$ = T and C and $S_{(strong)}$ = G and C. The second trace is the sequence of the HLA-B*44 allele generated by a GSSP.

The typing result of analyzing only exon 1, 2, 3, and 4 generic sequencing traces is shown in Figure 20.4c; 1,130 *genotypes* match with the data shown (data not shown), of which many only involve intron variants. In this figure, optimization is applied only to reporting differences at the two-field level. Notice that there are eight genotype ambiguities reported, of which seven are B35, 44 and one is B53, 44. In addition, 14 B44 alleles cannot be distinguished from each other. When the DNA sequences of exons 5, 6, and 7 are determined, it will resolve all allele ambiguities. Adding sequencing reactions specific for this example, the B44 alleles in exons 2 and 3 resolve all genotype ambiguities as shown in Figure 20.4d.

As can be seen in this example, multiple sequencing reactions are necessary to achieve high-resolution typing. Especially, the group-specific reactions needed to resolve the genotype ambiguities cannot be predicted.

20.11.2 NGS-based SBT Data Analysis

20.11.2.1 Introduction

NGS data contain highly detailed data of the genes amplified. With the correct software, determining the HLA genotype of a sample is in most cases straightforward. An important question is how reliable the HLA typing results are. In general, the results with the up-to-date protocols and software can be trusted, but especially when typing patients and/or potential donors, one should scrutinize the results to prevent wrong conclusions caused by artifacts. Software packages are – in

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general – optimized to give access to the typing results, the data, and the quality metrics describing the reliability. It also indicates what may be inspected in case the quality metrics do not meet the acceptance criteria. In this chapter, results obtained with NGSengine are shown.

20.11.2.2 The Data

NGS sequencers generate fastq files. Such a file contains all reads from a single sample. These reads may originate from multiple genes. Depending on the PCR and library preparation, the reads of 11 HLA loci of a single sample typically contain a few hundred thousand reads. A fastq file generated by a long-read sequencer contains a lower number of reads, since less reads are needed to cover a gene. A read consists of the DNA sequence of a single DNA fragment. For each nucleotide, a quality value (QV) is calculated in addition, which represents the reliability of the basecalling of that actual nucleotide (see Figure 20.15).

For the genes to be analyzed, the DNA sequences of the reported alleles are necessary. For HLA typing, these are based on the IPD-IMGT/HLA database[8] [7]. The resolution of the typing results also depends on the version used: using the newest version will allow correct identification of the most recently submitted and curated alleles. It also may lead to some extra ambiguities. For the stem cell transplant situation, one should use a recent version.

Software may also add expert knowledge to the results such as the Bw4/Bw6 status, serological equivalents, the CWD status, or the expected DRB1 – DRB345 associations.

20.11.2.3 Basic Analysis

Most software will follow the following steps from raw data to the HLA typing results:

- Determining which genes are covered by the reads in the fastq file.
- Aligning each read to the correct gene and the correct location.
- Phasing of the nucleotides at the heterozygous positions.

```
@M04735:275:000000000-K5M74:1:1101:14414:2537 1:N:0:162
ATAAGATCCTTCTTTAAACAAGAGAGGCATTAACTTGGAATGTCCGTGGAAAGTCTTAGAAGCTGATTGGTGTA
+
ABBABFFFFFFFGGGGFGGGGGGHHHGFGGHHHHHHHHHHHGFHHHHGGFGGFHHHHFHHHHHHHGHHGHHHFFFG
@M04735:275:000000000-K5M74:1:1101:11149:2552 1:N:0:162
GTGAGGATGGACCCAGTGGCCTCCCTAGTATCTCCTTTCTTTTCTTTCTGAACTCCAATGTTTATAAAGCCTGT
+
CCDCCFBFFCFFGGGGGGGGGGGHHGHHHHHHHHHHHHHHHHHHHHHHHHHHGHHHHHHHHGHHHHHHHHHHHH
@M04735:275:000000000-K5M74:1:1101:18795:2552 1:N:0:162
ACAGCAGGGGGCACTTAGGCTTCCTAGTCTGAGGGTGGCAGAGAGGCCCTCTCATCCCTTCCAGTTGGGCTACA
+
ACCCCFFABCCCGGGGGGGGGGHHHGHHHHHHHGGGGGHHGGHHGGGGGHHHHHHHHHHHHHHHGHHHHGHHHHH
```

Figure 20.15 Content of a fastq file. Each read consists of 4 lines, Line 1 starting with @ is the read name with additional information. Line two is the actual nucleotide content. Line 3 contains a + sign. Line 4 represents the encoded quality value. A fastq file can have an unlimited number of reads.

8 www.ebi.ac.uk/ipd/imgt/hla/

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- Calculate the typing results, which are the genotype(s) matching the data at best.
- Calculate quality metrics that assist the user in deciding about the reliability of the result.

After this, the user can inspect and, if necessary, modify the result:

- The user may edit to unclear basecalls.
- The user can approve or reject a result.
- The results will be uploaded to a LIMS.

Specifying some aspects of these steps:

- *Determining which genes are covered by the reads in the fastq file.*
  - Homology between gene sequences may influence correctness. Some alleles have larger regions identical to some alleles of other genes (see Figures 20.2 and 20.3). In these cases, it may not be possible to determine the correct locus for a small part of the reads. Ignoring some regions in the analysis may be necessary since homology may even be allele specific. As an example, when using short reads, exon 4 of DRB3 cannot be analyzed since some DRB3 alleles show high homology with DRB1*08 alleles. For long reads, this issue does not occur.
- *Aligning each read to the correct gene and the correct location.*
  - In most cases, alignment will result in a correct alignment. However, some regions, mostly located in introns, are difficult to align to. These regions involve alleles with homopolymers, long short tandem repeat (STR) stretches, or complex patterns of insertion or deletions (InDels), especially when alleles exist which differ in homopolymer or length of an STR. In DQA1, for example, at a location in intron 1, alleles have a stretch of A's of lengths 7–20. In DRB1 intron 2, just downstream exon 2, an STR is present in lengths of 15–31 times GT.
  - To enable more easy interpretation, some of these regions are excluded from routine analysis. This may result in ambiguities of intron variation.
- **Phasing:** Phasing is essential for high-resolution HLA typing and is explained elsewhere.
  - Lack of phasing will not give wrong typing results, but may increase the number of ambiguities. Lack of phasing can be caused by the following.
    - **Low read depth**: A minimum number of reads covering multiple heterozygous positions is needed for reliable phasing.
    - If the distance between neighboring heterozygous positions is larger than the maximum length of reads, no phasing between these positions is possible. Using long reads mostly eliminates phasing issues at all.
    - If the gene is not amplified in a single PCR, heterozygous positions lying in different amplified regions cannot be phased.
- **Genotype determination**: Based on the aligned and phased NGS data, the best-matching genotypes are determined.
  - An exact match is found when the best matching genotype(s) have zero mismatches with the data.

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- Also, near-matches are determined. In case the best match involves a rare allele, the near-matches allow inspection of the data at positions where the rare alleles differ from common alleles, which enables easy confirmation of the result by inspecting the data at those positions.

20.11.3 Analysis Result

Figure 20.16 shows a typing result of a single sample as generated by NGSengine®, developed by GenDx. Besides the typing results, quality metrics are shown. Although software packages may show quality metrics in a different way, these will allow the user to access their values.

The most important quality metrics are described below:

Sample mappability: The sample mappability in the upper-right corner of Figure 20.16 shows the percentage reads that could be assigned to any locus considered. This percentage should be high, but will not be 100%. There are three major causes for this: (i) the sequencer generated very short reads, probably originating from only barcodes; (ii) reads do not originate from HLA genes and are impurities of the amplification; and (iii) the reads can be assigned to multiple HLA genes, because they are small, or from a highly homologous region (see Figure 20.2a). The sample mappability should be stable and as high as possible.

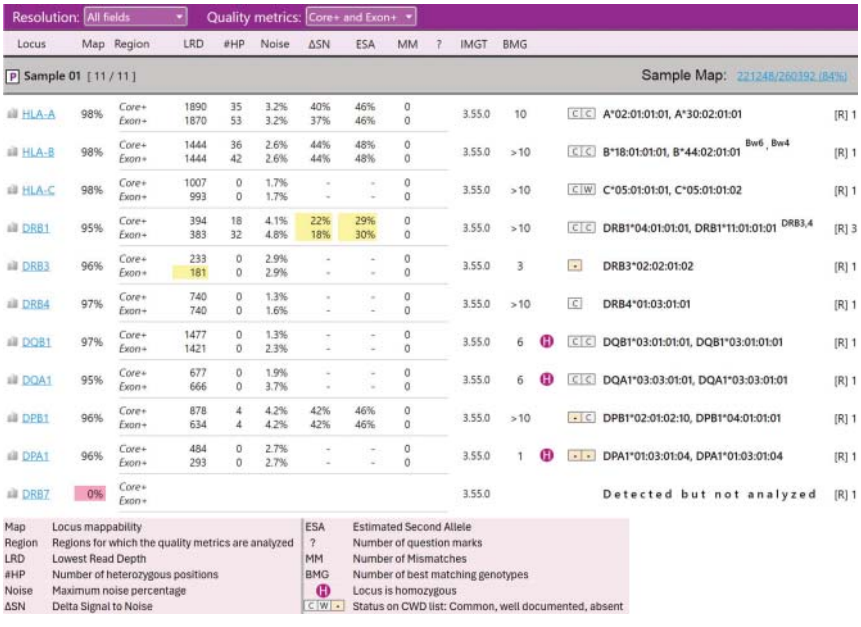

Figure 20.16 Typing result of a sample. For each locus, the HLA alleles are shown best matching the data and the quality metrics describing the data. The legend describes the abbreviations. Quality metrics that are sub-optimal are marked in yellow. Notice that for all loci, one or more exact matches are identified.

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In time, decreasing values may indicate issues with, e.g. sequencer or thermal cycler. In the example, sample mappability is 84%.

Locus mappability (Map): It is the percentage of reads assigned to a locus that ultimately was aligned in an acceptable way. The value is dependent on the complete workflow. It is important to monitor mappability: the value should be stable in time. In this example, the values are 95% and higher. A low locus mappability mostly indicates that the wrong analysis settings are used: reads have been found which map to regions not included in the alignment.

Lowest read depth (LRD): The number of reads covering a nucleotide position decides how reliable the basecalling can be done. The lowest read depth represents the position with the **minimum** number of reads. Every position is covered by at least this number. In Figure 20.16, the value is calculated for the core regions and all exons, allowing separate inspection of these selections.

Number of heterozygous positions (#HP): This number is a characteristic of the two alleles present in the sample. In HLA, this number can be low, but also very high. When this number is low, e.g. 1 or 2, extra inspection may be necessary since an isolated heterozygous position may, although rare, represent a single position with high noise. In case this number is 0, the locus is:

- **Homozygous**: The two alleles are identical.
- **Hemizygous**: Only one haplotype contains the gene, as can be the case for HLA-DRB3, HLA-DRB4, or HLA-DRB5.

Noise: In Figure 20.16, the value shown is the **maximum** noise identified in the region considered. Any position in this region will have a value equal to or lower than this value. Values as shown in the example are low. Causes of high noise can be:

- Low quality of the sequencing data.
- A sequencing artifact resulting in a few positions with high noise.
- A single heterozygous position that is not recognized and considered noise.
- Homopolymer or large STR regions that are difficult to sequence and analyze.
- Out-of-balance amplification. In case one allele is amplified in much lower efficiency compared to the other, the signal of this allele may completely or partly be missed, but will show itself as high noise.
- A gene missed by the locus finder. Due to the homology between genes, reads that originate from a gene not considered during analysis can be assigned to one of the other loci. Also reads that originate from pseudogenes may be aligned to a classical HLA gene.
- Analysis of a chimeric sample. Most software packages are designed to identify haploid genotypes. The presence of a third allele may be represented as high noise.

Estimated second allele (ESA): It represents the balance of the two alleles present in the sample. Ideally, this number is 50% but it should reliably be between 40% and 50%. In case of unbalanced amplification, this number can be much lower. With high-quality data, reliable HLA typing is still possible. In Figure 20.16, the ESA for HLA-DRB1 is 30%. However, the typing result is highly reliable, since the noise is below 5%. The reason for unbalanced amplification can be multiple.

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Delta signal to noise (ΔSN): The distance between the signal at the position with the highest noise and the signal at the position with the lowest heterozygous index indicates how accurately the two alleles can be identified. This number should be high. Low numbers can be caused by:

- Unbalanced amplification. In high-quality data, ΔSN will have a value in the same range as the ESA, but always lower.
- There is at least a single position where a heterozygous position is called homozygous.
- There is at least a single position where a noise artifact is present.

Number of mismatches (MM): This will be zero when the data match with at least one genotype. Mismatches should be inspected, and the nature of mismatches should be investigated. Reasons for mismatches can be:

- An unreliable basecall. The basecall should be corrected, or the position should be ignored for further analysis. If the position is within the core regions, or the exons, correction is preferred whenever possible. Positions in introns are less important for clinical use and may be ignored without accurate investigation.
- The position is located of which the allele sequence is not actually known, but imputed.
- The sample contains a new allele. In this case, it is important to evaluate the impact of a new polymorphism:
  - If it is located in a coding region or splice site:
    - Does it lead to a change of amino acids?
    - Can the new allele be expected to be a null allele either by a new indel, an introduction of a stop codon, or by an altered splice site?
    - It is a silent or coding difference with a known allele?
  - Is the new polymorphism only present in the intronic regions?
    - No change to the HLA proteins is expected.
  - The impact in the typing report should be included.

Number of best matching genotypes (BMG): This indicates the ambiguity rate for the locus. In most software, one can restrict the resolution to which reporting should be restricted. If there are any ambiguities at the two-field level, inspection is necessary since the ambiguities involve amino-acid differences in the alleles involved. In most cases, the ambiguity is caused by alleles differing in regions not included in the amplification or analysis. An example is that in older typing systems for DQB1, exon 4 was not included. The alleles DQB1*05:02:01:01 and DQB1*05:165 differ by one nucleotide in exon 4, which leads to an amino-acid difference. Many similar examples can be found also for other HLA genes: recently, DRB4*01:134 is recognized, which differs in exon 1 from DRB4*01:03:01:01. The two alleles, which differ by one amino acid from each other, only can be distinguished when exon 1 is included in both the amplification and the analysis.

20.11.3.1 Missing Allele Sequences and Sequence Imputation

Missing sequences in parts of the alleles is a problem for HLA typing. Although in general the allele sequences for the core regions are known for all alleles, this does not account for the other exons and some other regions [8]. In addition, for many

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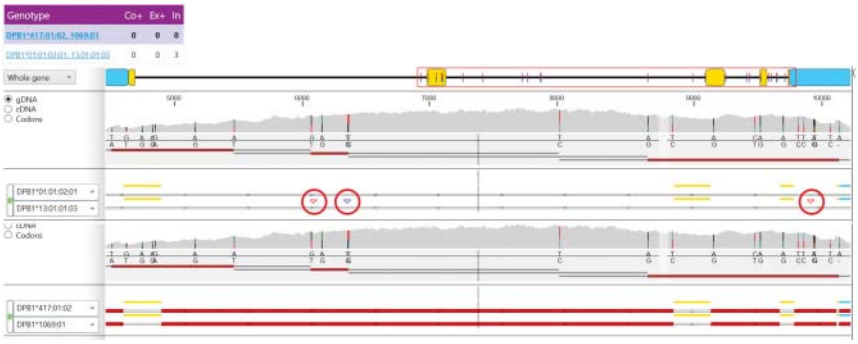

Figure 20.17 Example of an HLA-DPB1 genotype ambiguity. The combination of rare alleles, DPB1*417:01,1069:01, is listed first, since it has no mismatches with the data. The common combination DPB1*01:01,13:01 has three mismatches in the intronic region indicated by triangles and circles. No sequence of the introns of the rare alleles is known as indicated by the red lines in the alignment, leading to "no mismatches" in the introns.

alleles, introns are not sequenced fully. For DQB1, for some alleles even exon 2 is not completely sequenced. The most extreme allele is DQB1*05:03:02, for which only 149 of the 271 nucleotides of exon 2 are known in IMGT/HLA release 3.56. The allele was first recognized in 1990, and the sequence is never extended to fully cover exon 2.

Missing sequences may influence the typing result. In Figure 20.17, an HLA-DPB1 typing result is shown. The result is a genotype ambiguity DPB1*01:01:02:01,13:01:-01:03, which has only 3 mismatches with the data in the intron regions, and DPB1*417:01:02,1069:01 without any mismatches. The last genotype is listed first, but the reporting of zero mismatches in the intronic region is because there is no sequence recognized for the two rare alleles. For HLA typing, imputing these sequences is necessary to prevent that genotypes with incomplete sequenced alleles have better matching scores than genotypes with complete sequenced alleles. The methods used mostly involve imputing the sequences from other alleles from similar allele groups with names sharing the first, second, and/or third field. In the vast majority, the results are reliable, but in rare cases, this may draw wrong conclusions. This stresses the need to submit the sequences of regions that are currently unknown for the alleles involved. Imputing unknown allele sequences is possible for all classical HLA genes, with the exception of HLA-DPB1.

20.11.4 Artifacts

NGS data generated by HLA typing protocols in general are of high quality. Analysis of NGS data for HLA typing encounters some challenges with respect to the genes and nature of the sequencing process. These artifacts may lead to high noise or unexpected patterns in the data. Some regions of the HLA genes are by default excluded from the analysis to prevent unnecessary focus and distraction from the clinically relevant information. However, before submitting the sequence of a new allele, the data should cover the whole gene without any gaps.

Some common artifacts are given as follows.

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20.11.4.1 Homopolymers

Homopolymers are stretches of identical nucleotides. In the HLA genes, they are especially present in introns. A fraction of the reads will not have the correct number of nucleotides that originally were present in the allele. In addition, alleles may have different lengths of homopolymers, as already described in the alignment section. This results in complicated alignment patterns which may not be interpreted with high confidence.

A particularly important homopolymer is located in HLA-A at the beginning of exon 4. This homopolymer contains in most expressed alleles 7 Cs (ACCC-CCCCAAG). In a number of alleles, the homopolymer has a different length. The homopolymer can have lengths of five in A*24:40N, six in four other alleles, eight in thirteen alleles including the A*01:04:01:01N, or nine in the A*30:130N (IPD-IMGT/HLA 3.57). All these alleles are considered null alleles. Determining the correct length of the homopolymer is important to determine the presence or absence of the null allele.

20.11.4.2 Short Tandem Repeats (STR)

A STR is a stretch of repeated short sequences. For instance, "GATGATGATGAT" is a 3-nucleotide STR of GAT. For longer STRs, PCR amplification may be inaccurate, and a fraction of the reads will not have the correct STR length. In addition, alleles may have different STR lengths. This results in complicated alignment patterns which may not be interpreted with high confidence. Some alleles may be difficult to discriminate reliably since they only differ in the lengths of the STRs.

As an example, in DRB1, about 50 bp into intron 2, a complex STR region is present consisting of multiple, sometimes interrupted, STRs, which also show a large variation in length. All patterns contain a $(GT)_m$ STR, in which m is the number of GT in a row. M ranges from 10 to 28 [9]. Other HLA genes may also have STR sequences fortunately located in the intronic regions.

20.11.4.3 Insertion or Deletion (InDel)

The sequences of alleles may have different lengths, especially in intronic regions. InDel describes a length difference in a region, since it may have originated not only as an insertion in a smaller allele but also as a deletion in the larger allele.

InDels can have any size from a single nucleotide to very long regions. Small InDels, if not part of an STR or homopolymer, will not influence the HLA typing accuracy. InDels with lengths of about the size of the read lengths and longer may result in ambiguous alignments, since it may be unclear if in a region where the data support homozygosity, the two alleles are identical or that one of the alleles has a large deletion. Complex regions often are a combination of STRs, homopolymers, and InDels. This hampers the alignment accuracy. Alignment of reads that start or end in such a complex pattern may become ambiguous.

InDels in exons in general will cause the allele to be a null allele, especially when the InDel introduces a frameshift. There are, however, exceptions: a prominent InDel, which does not silence the alleles, is located in the middle of exon 2 of HLA-DQA1. In 325 (41%) alleles, codon 56 shows a 3-basepair deletion compared to 459 (59%) alleles in IPD-IMGT/HLA 3.55.

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20.12 Special Cases

The power of NGS data is not only beneficial for HLA typing. The data also may give insight into valuable patient information, which go beyond determining the HLA type of a sample. Some examples are the detection of new alleles, indications for loss of heterozygosity (LOH), indications for allele-specific mutations, or indications for a chimeric situation. In this section, some examples are given.

20.12.1 New Alleles

From new alleles by NGS, the DNA sequence is available in detail, which allows for direct description if the allele differs in protein from the closest match.

A new allele present in a sample for a particular locus is in general easily recognized, since there will be no genotype with an exact match (see Figure 20.18). Software should show where the differences are with the exact match and allow inspection of the quality.

Especially if the transplant typing concerns a potential donor or recipient, it is important to determine if the new allele also encodes for a different protein in comparison with the best match. Criteria to determine are:

- Could the new allele be a null allele?
  - The new polymorphism encodes for a stop codon.

Figure 20.18 The results of an HLA-C analysis. The red marking in the typing report indicates that the data does not match with the best matching genotype. There is a single base mismatch that is located in the core region. The base variation plot shows that the data are of excellent quality. The best matching genotype differs at a single position in exon 3, as indicated by the triangle in the circle. The mismatch between data and the best match is present in the C*07:01:02:01 allele. The mismatch position does not introduce a new amino acid in comparison with the common allele (not shown). Reanalysis with IPD-IMGT/HLA version 3.55.1 revealed that the new allele has been submitted and assigned C*07:01:113.

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- There is an InDel in the new allele causing a frame shift.
- A splice site modified is expected to result in alternative splicing.

- Does the amino acid sequence differ?
  - The polymorphism results in a codon differing from the one in the best match.

If the new allele is expected to result in a null allele or a protein differing from the best match, this should be reported to the clinicians, since it may influence the matching procedure.

In the past, alleles have been discovered which did not follow the rules described. For instance, the allele sequence of the null allele A*01:11N is identical to the sequence of the A*01:01:01:01 for all exons and introns, except for a single SNP in exon 3, which according to the codon usage was noncoding [10]. However, the expression of the allele was absent with multiple techniques tested. The new SNP introduced a new dominant splice site as was confirmed by sequencing the mRNA. The change prevented the generation of a mature HLA protein.

Sequences of new alleles are expected to be submitted to the IPD-IMGT/HLA database according to their criteria. Future recipients possessing this allele may benefit from finding a match.

20.12.2 Mutation and Loss of Heterozygosity (LOH)

HLA LOH is increasingly being recognized as an important immune escape mechanism in response to therapy [11–13]. LOH is a common genetic event in the development of cancer, but also after transplantation for the non-matched genes or haplotype. Mutation silencing the expression of a gene or an allele is a second mechanism to escape the immune response.

Mutations in the HLA genes may be detected by the NGS data. Although the data never prove the actual mutation, they may support clinicians in their clinical diagnosis, and in specific cases, they suggest that the alleles involved are silenced. Figure 20.19 shows the data of an HLA-C typing of a sample. One position in codon 54 shows aberrant statistics. A fraction of the reads has the sequence of TAG, a

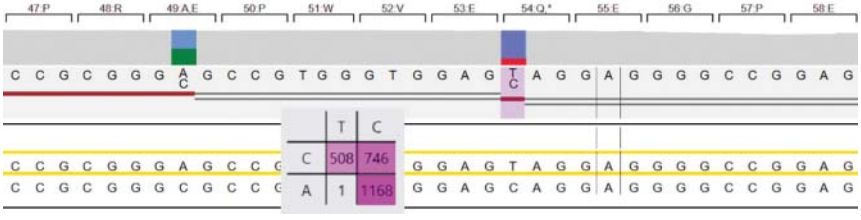

Figure 20.19 A partial sequence of exon 2 of HLA-C. The phasing data between the heterozygotes at codon 49 and 54 show that at codon 49, the ratio CA is 1254:1169 = 52% and 48%. This reflects a balanced amplification of the two alleles. All reads of the allele with A at codon 49 have a C at codon 54. However, of the reads with a C at codon 49, 41% (508/1254) has a T at codon 54, and 59% have a C at codon 54. A possible reason is that the sample is a mixture of normal and tumor cells. In the tumor cells, the allele with a C at codon 49 has a C to T mutation at codon 54, converting the codon from CAG to TAG, which is a stop codon. By this mutation, the allele is silenced.

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stop codon. However, the distribution does not follow a normal two-allele pattern, especially when the phasing statistics with the heterozygous position in codon 49 is inspected. The pattern matches a situation of a sample that is a mixture of healthy cells and cells in which one HLA-C allele is mutated and by this mutation is silenced. If there is suspicion that this is a mutation in part of the cells, sequencing a sample from a different source, e.g. a buccal swab in which this aberrant pattern is not present, confirms that the aberrant pattern is not a technical artifact and represents a mutation in part of the cells.

LOH of HLA genes also may be detected by NGS data. If NGS shows an unbalance of two alleles involved, the most likely explanation is that the primary PCR shows unbalanced amplification. However, LOH of the gene may also cause that the two alleles are not represented in the data with the same fraction. In particular for loci where the allele balance is always stable, this may be an indication that there is LOH. In some cases, multiple loci show unbalanced data, suggesting that LOH involves a larger part of the MHC region. The conclusion that LOH is present may be confirmed if data from a sample from another source of the same person, e.g. a buccal swab, show a balanced presence of the alleles.

20.12.3 Chimeric Sample

If the HLA genes of chimeric samples are sequenced, the NGS data patterns may look confusing. However, with the proper bioinformatics methods in many cases, the alleles present can be determined. Samples taken post-transplant may appear chimeric. If properly analyzed, a mixture of the alleles from the recipient and donor can be identified. After haploidentical stem cell transplantation, for each gene, the shared allele is present in all cells, and the ratio between the identified donor and recipient-specific allele reflects the chimeric state. Rarely, chimerism also is detected in SCID patients undergoing multiple blood transfusions [14].

Abbreviations

HLA	human leukocyte antigen
GvHD	graft versus host disease
SBT	sequencing-based typing
NGS	next-generation sequencing
SSP-PCR	sequence-specific primers
PCR-SSOP	sequence-specific oligonucleotides probes
PCR	polymerase chain reaction
dNTP	deoxyNucleotide TriPhosphate
ddNTP	dideoxyNucleotide TriPhosphate
GSSP	group-specific sequencing primers
MSP	motif-specific primers
HARP	heterozygous ambiguity resolving primers
SBS	sequencing-by-synthesis
DNB	DNA nanoballs

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RCR	rolling circle replication
RCA	rolling circle amplification
SMRT	single molecule real time sequencing
ZMW	zero-mode waveguide
ONT	oxford nanopore technology
ESA	estimated second allele
ΔSN	delta signal to noise
BMG	best matching genotype
STR	short tandem repeat
InDel	insertion or deletion
LOH	loss of heterozygosity
UTR	untranslated region
Locus	a physical site or location within the genome

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21

Crossmatching in Transplantation

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21.1 Introduction

Crossmatching is a crucial procedure in medicine that ensures the compatibility of donor and recipient tissues [1]. Its primary goal is to assess the compatibility between a recipient and a potential donor by detecting the presence of preformed immunological processes that could lead to graft rejection. The detected compatibility has impact on the decision of clinicians to accept a specific organ offer. Historically, it was because of the lack of other precise methods the only method to test the compatibility between donor and recipient. Since then, the practice has evolved significantly, particularly with advancements in immunology, serology, and molecular techniques.

21.1.1 Importance of Crossmatching

Early transplantation efforts faced high rates of graft rejection, primarily due to incompatibility between donor and recipient. The development of crossmatching tests was a pivotal moment as it allowed clinicians to better predict and avoid complications like hyperacute rejection (HAR) and to improve long-term graft survival. HAR is a rapid and often fatal immune response that occurs when a recipient's preexisting antibodies react with donor antigens, leading to immediate graft failure. It was demonstrated that 80% of patients who underwent transplantation with a positive complement-dependent cytotoxicity crossmatch (CDCXM) experienced immediate kidney allograft failure, compared to only 15% of patients transplanted with a negative CDCXM [1]. This made the CDCXM an essential and mandatory test for kidney pretransplant evaluation to avoid potential HAR mediated by antibodies [1, 2].

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21.2 The Mechanism of Crossmatching

21.2.1 Immunological Basis

A crossmatch test determines the immunologic risk of organ rejection by ensuring that there are no transplant-relevant circulating antibodies in the recipient directed against donor antigens which could cause antibody mediated rejection. Donor-specific antibodies (DSA) in the recipient serum may exist prior to transplantation (preformed donor specific antibodies/DSA) or develop as a consequence of sensitization to the mismatched donor HLA antigens (*de novo* DSA). DSA binding to donor HLA on the endothelial surface has a number of potential consequences [3–5]. Crossmatching relies on the principle of antibody-mediated recognition of antigenic differences between the recipient and donor. The key antigens involved in crossmatching in the field of histocompatibility are the Human Leukocyte Antigens (HLAs) which play a central role in immune responses.

21.2.2 Types of Physical Crossmatching

To date, standard practice is still the physical crossmatch (PXM) test, such as CDCXM or flow cytometry crossmatch (FCXM). Furthermore, it is possible to do an "in silico" crossmatch, the so-called virtual crossmatch (VXM).

There are two main types of serological or PXM tests:

1) **Complement-dependent cytotoxicity (CDCXM) crossmatch:** This test involves mixing the recipient's serum with donor lymphocytes and observing for complement-mediated lysis of the donor cells.
2) **Flow cytometry crossmatch (FCXM):** A more recent technique, flow cytometry allows for a more sensitive detection of anti-HLA antibodies, including those that may not activate complement.

21.3 Crossmatch Methods

21.3.1 CDC Crossmatch

The complement-dependent cytotoxicity (CDC) crossmatch is a critical laboratory technique employed in transplant immunology to evaluate the compatibility between a donor and recipient. Despite being one of the older methods, the CDC crossmatch remains a cornerstone in pretransplant testing due to its proven reliability and clinical relevance.

Introduced in the 1960s, the CDC crossmatch was the first assay to effectively predict transplant outcomes based on immunological compatibility. It significantly reduced the incidence of HAR, thereby improving graft survival rates. Over the

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years, the CDC crossmatch has evolved with enhancements such as the anti-human globulin augmentation to increase its sensitivity. Despite advancements in other crossmatching techniques, the CDC crossmatch remains widely used due to its simplicity, cost-effectiveness, and ability to detect clinically significant DSAs [6].

21.3.1.1 Principle of CDC Crossmatch

The CDC crossmatch test is based on the principle of complement activation and cell lysis. The procedure involves mixing the donor's lymphocytes with the recipient's serum. If the recipient has preformed antibodies against the donor's HLA, these antibodies will bind to the donor lymphocytes. When complement is added to the mixture, it gets activated by the antigen-antibody complexes, leading to the lysis of the donor cells. The extent of cell lysis is then assessed microscopically. A positive CDC crossmatch indicates the presence of cytotoxic antibodies that can cause HAR of the graft.

In the CDC crossmatch, three components are brought in a Terasaki chamber into contact with each other.

1) **Serum of the recipient**: The serum of the recipient might contain antibodies which are directed to the donor's organ. Preformed antibodies can originate from a former immunization of the recipient as for example blood transfusions, former transplantations or pregnancies.
2) **Living cells of the potential donor:** Lymphocytes of the donor are taken from a blood draw or sometimes out of the vasculature of the spleen.
3) **Complement:** In most cases, rabbit complement is used due to the availability and to the most similar behavior in the human antigen antibody reaction.

In case of a successful binding of the antibodies to targets on the cell surface, the complement cascade helps to form the membrane attack complex, which kills the target cells.

The detection of dead cells is done by a staining formerly with eosin, in our days with a couple of fluorescent dyes coloring living and dead cells differently.

The combination ethidiumbromide/acridinorange is used, where the dead cells are stained by the membrane impermeable ethidiumbromide (red–orange) and the living cells actively take up acridinorange (green).

Under a microscope, the ratio of living and dead cells is monitored and score is given. (1–8, 1 no dead cells; 8 100% dead cells). In case of more dead than living cell, the crossmatch is called "positive"

To get an additional insight in the ongoing process, the lymphocyte population can be separated into B- and T-lymphocytes allowing to differentiate if more HLA class 1 or HLA class 2 antibodies are involved. And with the help of DTT, it is also possible to distinguish between IgG- and IgM antibodies (see also the antibody detection chapter) [6].

Protocol of CDCXM [6].

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Textbox 21.1 Standard CDC Crossmatch.

Tray Set-Up

1. Add 1 µl of ATG dilutions and AB negative sera to an oiled Terasaki tray using a Hamilton syringe
   3A—ATG50, 3B—AB Neg, 3C—ATG500, 3D—ATG200, 3E—AB Neg, 3F—B Cell Pos, 4A—ATG400, 4B—ATG25, 4C—AB Neg, 4D—ATG100, 4E—ATG800, 4F—ATG1000.
2. Add 1 µl recipient serum to wells 1D, 1E, and 1F and 1 µl AB negative serum to wells 1A, 1B, and 1C using a Hamilton syringe. Historical peak sera can be added as appropriate in triplicate to wells 2A to 2F. Trays should be made at least in duplicate for both T and B cell crossmatch, i.e. at least four trays in total.

Crossmatch Set-Up

1. Add 1 µl T or B cell suspension using an automated cell dispenser to avoid cross-contamination. Ensure all wells are thoroughly mixed.
2. Incubate the trays for 45 minutes at 22 °C (or room temperature).
3. Following incubation, add 5 µl of previously screened pooled rabbit complement to each well using an automated dispenser to avoid cross-contamination.
4. Incubate the trays for 45 minutes at 22 °C
5. Following complement incubation, add 5 µl hemoglobin/ethidium bromide stain to each well. Allow cells to settle for approximately 20 minutes.
6. Score the percentage cell death semi-quantitatively under the fluorescent microscope according to the following table.

Cytotoxicity scores

Percentage cell death	Score	Conventional score
0	A	1
1–2	B	1
3–5	C	1
6–10	D	1
11–15	1	1
15–25	2	1
25–35	3	2
35–45	4	2/4
45–55	5	4
55–65	6	4/6
65–75	7	6
75–85	8	8
85–100	9	8
Empty well or unreadable	X	0
Insufficient cell numbers	0	0

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Interpretation of Crossmatch Results

1. A positive or negative crossmatch is determined by comparing the crossmatch score with the background reactivity, i.e. the AB negative control serum
2. Results should be interpreted according to the table "interpretation of CDC crossmatch results".
3. Results are no longer considered purely as an indication/contraindication to transplantation; but are reviewed with other immunological data to identify level of risk

Interpretation of CDC crossmatch results

Autologous T cell crossmatch result	Autologous B cell crossmatch results	Allogeneic T cell crossmatch results	Allogeneic B cell crossmatch results	Interpretation
Negative	Negative	Negative	Negative	Low-risk transplant, not a contraindication
Negative	Negative	Negative	Positive	Moderate-risk transplant, not an absolute contraindication Suggests donor-specific HLA class II antibodies
Negative	Negative	Positive	Positive	High-risk transplant, possibility of hyperacute or accelerated rejection, contraindication
Negative	Negative	Positive	Negative	Potential high-risk transplant, however, is an unusual result for HLA antibodies, as the B cell crossmatch should be positive. Warrants further investigation for non-HLA antibody
Negative	Positive	Any result	Any result	Removal of IgM is required prior to crossmatch
Positive	Positive	Any result	Any result	Removal of IgM is required prior to crossmatch

CDC = complement-dependent cytotoxicity; HLA = human leukocyte antigen.

21.3.1.2 Limitations and Challenges

Despite its importance, the CDC crossmatch has several limitations. It has a lower sensitivity compared to more modern assays such as FCXM and solid-phase assays. Additionally, the interpretation of CDC crossmatch results can be subjective, and the assay may produce false-negative results, particularly in cases where the antibodies do not fix complement efficiently. False positive reactions can be caused by cytotoxic substances in the patient serum (e.g. cytotoxic drugs), or improper isolation, or damaged donor cells [7].

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21.3.2 Flow Crossmatch: An Essential Tool in Transplant Immunology

Flow crossmatch (FCXM) is another laboratory technique used in the field of transplant immunology to detect preformed DSAs. FCXM is much more sensitive than CDCXM and can therefore detect lower concentrations of DSAs in the recipients' serum [7–9].

21.3.2.1 Principle of Flow Crossmatch

FCXM utilizes flow cytometry, a technology that allows for the detailed analysis of cell surface markers using fluorescently labeled antibodies [7, 10]. In the context of organ transplantation, the assay involves mixing the donor's lymphocytes (T cells and B cells) with the recipient's serum. To this part, the same as in the CDC crossmatch. Because of the different endpoint detection method complement is not necessary. If the recipient's serum contains specific antibodies against the donor's HLA, these antibodies will bind to the donor's lymphocytes. Subsequent incubation with fluorescently labeled anti-human immunoglobulin (IgG) antibodies allows for the detection of these bound anti HLA-antibodies via flow cytometry [9].

Textbox 21.2 Halifax FCXM Protocol.

The Halifax FCXM protocol was performed in 96-well U-bottom BD Falcon Microplate trays. 50 μl of test or control sera and 25 μl of enriched donor PBMC (2.5×105 cells) were added to each reaction well in a 96 well tray, dry vortexed, and incubated at RT for 20 minutes.

Cells were washed three times with 200 μl FWB at $500 \times g$ for 1 minute, after which antibody cocktail (3 μl of anti-CD3-PerCP, 2 μl of anti-CD19-PE, 0.25 μl of anti-IgG-FITC, made up to 100 μl with PBS) was added to the cells, dry vortexed, and incubated for 10 minutes at RT in the dark.

Cells were washed twice more with 200 μl FWB at 500×g for 1 minute, resuspended in 150 μl FWB, and transferred into 5 ml polystyrene Falcon tubes containing an additional 250 μl of FWB before acquisition on the flow cytometer.

Flow cytometry data were acquired using the FACS Canto II flow cytometers (BD Biosciences) and analyzed with BD FACSDIVA™ software (BD Biosciences) on the 1024 channel scale.

Optimization experiment crossmatch data were expressed as the median channel fluorescence shift (MCFS) from the NC serum. Validation experiment crossmatch data were expressed as MCFS from the 3-standard deviation (3SD) cutoff, determined using the negative patient sera FCXM median channel fluorescence (MCF) values. Halifax HLA laboratory 3SD cutoffs for the Halifax FCXM protocol are shown in Table 1 and compared to the standard protocol (SFCXM) from Liwski et al. [11]. The positive FCXM cutoffs used by the SWID HLA laboratory were >77 and >89 MCFS for T and B cells, respectively. The positive cutoffs used by the Santa Casa Laboratory were >77 and >113 MCFS for T and B cells, respectively.

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Table 1

Parameter	T cell FCXM		B cell FCXM	
	Standard	Halifax	Standard	Halifax
Negative control, mean MCF (SD)	168 (21)	168 (18)	216 (25)	215 (25)
Negative crossmatch patient sera, mean MCF (SD)	161 (26)	158 (25)	213 (32)	203 (34)
3SD cutoff value, MCFS	79	74	97	101
Mean crossmatch cutoff, MCF	247	242	313	316

Source: Adapted from Liwski et al. [11].

21.3.2.2 Advantages Over Traditional Techniques

Traditional crossmatching methods, such as the CDC assay, have been widely used but possess certain limitations. The CDC assay relies on the activation of complement to lyse donor cells in the presence of specific antibodies, which can lead to false negatives due to its lower sensitivity and inability to detect non-complement-fixing antibodies. In contrast, FCXM offers superior sensitivity and specificity, enabling the detection of both complement-fixing and non-complement-fixing antibodies, thereby reducing the risk of transplant rejection due to undetected DSAs. Other advantages are the ability to measure separately B- and T-cell specific antibodies by using additional channels of the flow cytometer and corresponding fluorescently labeled anti-B- or T-cell characterizing antibodies (CD19 for B-cells, CD3 for T-cells). In addition, it is possible to look for the presence of IgM DSAs by using a labeled anti-IgM detection antibody in an additional fluorescence channel. You potentially can determine all of these parameters in one sample without separating the cells reducing the risk of cell damage [10, 12, 13].

Very important as in all other crossmatch protocols is a rigorous control and standardization regime [14].

21.3.2.3 Limitations and Challenges

Despite its advantages, FCXM is not without limitations. The technique requires specialized equipment and technical expertise, which may not be readily available in all transplant centers. Additionally, the interpretation of FCXM results can be complex, necessitating correlation with clinical findings and other immunological assays [15, 16]. Nevertheless, in North America FCXM has become the standard procedure.

21.3.2.4 Conclusion

FCXM is a pivotal assay in the realm of transplant immunology, offering enhanced sensitivity and specificity for the detection of donor-specific antibodies. Its ability to identify high-risk recipients has significantly contributed to the improvement of transplant outcomes. As technology advances, further refinement of FCXM and its

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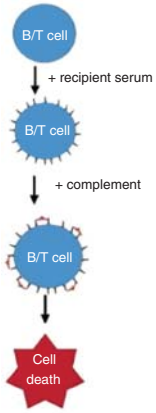
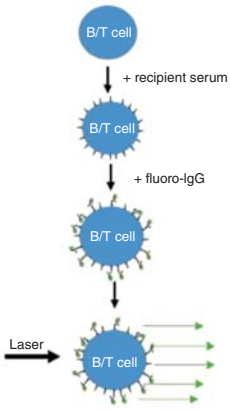
Table 21.1 Comparison of antibody testing methods.

Antibody test methods	CDC crossmatch	Flow crossmatch
Diagram of protocols		
Usage of test	Usually pre-transplant	Usually pre-transplant
Antigen source	Native antigens on donor lymphocytes	Native antigens on donor lymphocytes
Antibody detected	Cytotoxic, complement-fixing IgG and IgM, donor-specific	Donor-specific IgG
Antibody specificity determined	No	No
Quantitation	Scale 1–8 binary	Semi-quantitative
Sensitivity	Low	Intermediate
Repeatability	Low	Low to intermediate
Requirement for live cells	Yes	Yes

CDC = complement-dependent cytotoxicity; HLA = human leukocyte antigen.
Source: Adapted from McCaughan et al. [17].

integration with other diagnostic modalities hold promise for even greater precision in pretransplant immunological assessment (Table 21.1).

21.3.3 Virtual Crossmatch

VXM is a sophisticated technique in transplant immunology that predicts donor–recipient compatibility without the need for physical mixing of donor and recipient cells and serum. Utilizing advanced immunological data, the VXM has revolutionized pretransplant testing, offering significant advantages in speed, sensitivity, and specificity [18, 19].

21.3.3.1 Principle of Virtual Crossmatch

The virtual crossmatch relies on comprehensive immunological profiling of both the donor and recipient, particularly focusing on the presence of HLA antibodies in the recipient's serum. The process involves comparing the recipient's known HLA antibody profile, obtained through solid-phase assays such as Luminex, with the donor's HLA typing. If the recipient has performed antibodies against any of the donor's HLA antigens, the VXM is considered positive, indicating a higher risk of antibody-mediated rejection [19].

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21.3.3.2 Advantages over Traditional Methods

One of the main advantages of VXM is its speed. Traditional crossmatching techniques like CDC and FCXM require the physical presence of donor cells and recipient serum, which can be time-consuming and logistically challenging. These samples also need to be regularly updated and replaced, as the recipient's antibody status may change due to events that cause a change in sensitization status, such as blood transfusions, infections, or placement of mechanical circulatory support, or as a result of treatment for desensitization. In contrast, VXM can be performed rapidly using existing immunological databases, making it particularly useful in scenarios where quick decision-making is crucial, such as in deceased donor transplants.

VXM also offers enhanced sensitivity and specificity. By using solid-phase assays to detect HLA antibodies, VXM can identify even low levels of DSAs that might be missed by CDC crossmatch. Furthermore, VXM eliminates the variability and subjectivity associated with interpreting PXM results, leading to more consistent and reliable outcomes [20].

21.3.3.3 Limitations and Challenges

Despite its many advantages, VXM is not without limitations. The accuracy of VXM depends heavily on the quality and completeness of the immunological data. Discrepancies in HLA typing or incomplete antibody profiles can lead to false-negative or false-positive results. It has to be taken into account that antibody profiles change and the antibody status is required to be up to date. Additionally, VXM does not provide information about non-HLA antibodies, which can also play a role in graft rejection.

Another challenge is the need for continuous updating and maintenance of immunological databases to reflect the latest advancements in HLA antibody detection and typing technologies. Ensuring that these databases are comprehensive and up-to-date is critical for the reliability of VXM [16].

21.3.3.4 Conclusion

Virtual crossmatch represents a significant advancement in transplant immunology, offering a rapid, sensitive, and specific method for predicting donor–recipient compatibility. It also enables the identification of highly sensitized patients who have antibodies against a broad range of potential donors, without the need for performing a high number of laboratory experiments under time shortage, thus facilitating better allocation of organs and reducing the likelihood of rejection [20, 21]. VXM also plays a crucial role in kidney paired donation programs, where it helps match donors and recipients more accurately, increasing the chances of successful transplantation (Table 21.2).

Studies have shown that VXM correlates well with traditional crossmatching techniques in predicting transplant outcomes. For instance, a positive VXM has been associated with a higher incidence of antibody-mediated rejection and lower graft survival, highlighting its clinical relevance in pretransplant risk assessment. As a result, many transplant centers have integrated VXM into their standard pretransplant evaluation protocols [19, 22].

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Table 21.2 Comparison of crossmatching tests.

Crossmatch	CDCXM	FCXM	VXM
Target	Donor's cells	Donor's cells	Interpretation of results from SAB and donor HLA molecular typing
Type of antigens	Native HLA on the cell membrane	Native HLA on the cell membrane	Recombinant HLA proteins
Type of antibodies detected	Cytotoxic IgG/IgM antibodies	IgG antibodies bound on the lymphocyte membrane	IgG antibodies bound to HLA on the solid phase (Luminex)
Specific for anti-HLA antibodies	No	No	Yes
Interpretation	Subjective:	Objective:	Objective:
	Operator-dependent technique	Based on the difference between the test serum MFI and the negative control	Based on the MFI of specific antigens
Sensitivity	Low	High	High
Approximate processing time	5 hours	3 hours	≤1 hour
Predicting the clinical outcome of transplants	Hyperacute rejection	Acute rejection	Acute/chronic rejection

CDCXM = complement-dependent cytotoxicity; FCXM = flow cytometry crossmatch; HLA = human leukocyte antigen; MFI = mean fluorescence intensity; SAB = single antigen bead; VXM = virtual crossmatch.
Source: [19]/Wolters Kluwer Health, Inc/CC BY 4.0.

21.4 Summary

The ability of crossmatch techniques to identify harmful antibodies has been instrumental in the successful transplantation of kidneys, hearts, and other organs. It provides crucial information that helps transplant teams in making informed decisions regarding donor–recipient matching and in planning desensitization protocols for sensitized patients.

The CDC crossmatch still plays a pivotal role in the organ allocation process. A positive CDC crossmatch is typically considered a contraindication to transplantation due to the high risk of HAR.

The implementation of FCXM has expanded the pretransplant compatibility testing for the identification of sensitized recipients with lower antibody levels. This information is crucial for clinical decision-making, including desensitization

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protocols and donor selection. Studies have demonstrated that recipients with a positive FCXM bear a higher risk of graft rejection and reduced graft survival compared to those with a negative FCXM.

The implementation of VXM took this principle further and decoupled the matching process from the lab accelerating the allocation process, where there is a need for a rapid decision. By leveraging detailed immunological data, VXM enhances the precision of pretransplant evaluations, contributing to improved transplant outcomes. As technology continues to evolve, the integration of VXM with other diagnostic tools holds promise for even greater accuracy and efficiency in the field of organ transplantation.

Overall, the crossmatch will remain a main tool for the final decision of accepting an organ offer, with new techniques promising a bright future of the crossmatch as diagnostic tool.

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22

Determination of Antibodies and Typing in the HPA System

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22.1 Introduction to Platelet Antibody and Antigen Testing

Human platelet antigens (HPA) are described in Chapter 6. Moreover, HLA class I antigens and the blood group determinants A and B are expressed on platelets. Platelet alloantibody testing is based on incubating different test platelets with known HPA antigens with the serum sample tested. Deducing antibody specificities from reaction patterns becomes difficult, if the serum contains mixtures of platelet reactive antibodies. This is particularly a problem with mixtures of HLA class I and "platelet specific" HPA antibodies. Assays for platelet reactive alloantibodies are required to discriminate HLA class I antibodies and "platelet-specific" antibodies as the great majority of cases with *fetal and neonatal alloimmune thrombocytopenia (FNAIT)* are caused by HPA-antibodies reacting with epitopes on GP IIb/IIIa, Ib/IX, Ia/IIa, and CD109. HLA class I antibodies are suspected to cause FNAIT only in rare exceptional cases. Similarly, the transfusion reaction of *post-transfusion purpura* is usually associated with antibodies against HPAs located on GP IIb/IIIa. Sera of thrombocytopenic patients who experience *febrile reactions following platelet transfusions* or who are *refractory to platelet transfusions* require investigation for both HLA class I and HPA antibodies, if immunization against platelet specific antigens and/or HLA antigens is suspected [1].

22.2 Principles of Antibody Testing

In the last decades, various laboratory methods for detection of platelet alloantibodies were developed (Table 22.1) [2]. **Platelet antibody tests** of the **first generation** include: *platelet agglutination*, *platelet complement fixation*, *^{14}C serotonin release assay* and *inhibition of clot retraction*. They are based on platelet activation or other secondary functional effects induced by antibody binding to platelets. The *lymphocytotoxic test (LCT)* is considered as surrogate test for detection of HLA class I

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Table 22.1 Development and principles of platelet antibody assays, for references, see [2].

	Principle	Examples of assays
First generation assays	Detection of secondary effects of antibody binding to platelets	Complement fixation test, platelet agglutination test, platelet serotonin release test, inhibition of clot retraction
Second generation assays	Qualitative or quantitative measurement of Ig binding to platelets	Platelet suspension immunofluorescence test (PSIFT), platelet radioactive anti-immunglobulin test, mixed platelet hemagglutination assay (MPHA), competitive enzyme-linked immunosorbent assay (quantitative), quantification of Ig in platelet lysates
Third generation assays	Detection of Ig binding to platelet glycoproteins	Radioimmunoprecipitation, immunobead assay, MAIPA assay, antigen capture ELISA (ACE), modified antigen capture ELISA (MACE) simultaneous analysis of specific platelet antibodies assay (SASPA); commercial assays: PakPlus[a], PakLx[b]

a) an antigen capture ELISA.
b) an immunoassay based on the Multi-Analyte Profiling (xMAP™)-Technology (Luminex™).

antibodies reacting with platelets. **Second generation assays** allow qualitative or semiquantitative assessment of immunoglobulin binding to platelets. Typical examples are *platelet radioactive anti-immunoglobulin test (PRAT)* [3], *mixed passive hemagglutination assay (MPHA)* [4], *platelet suspension immunofluorescence test (PSIFT)* [5].

The PSIFT [5] is considered the reference method among second generation assays, a detailed protocol is given below. A commercial assay (*Capture-P®*, Immucor), is based on visual examination of Anti-IgG-coated indicator cells in wells of microtiter plates coated with donor platelets. The wells are first incubated with the patient's serum, washed and the indicator cells are added. They will adhere to IgG bound to the immobilized test platelets. This assay yields positive results with HLA class I and HPA-antibodies. It is marketed in a variant for antibody screening and in another variant for platelet crossmatching. However, in a single-center study over more than a decade, the sensitivity of this assay seems to have changed [6]. Another commercial assay for platelet crossmatching which follows a similar principle is MASPAT(R) (Essange Reagents, The Netherlands).

Third generation assays allow to detect binding of antibodies to platelet (glyco)proteins (GPs). The technique of *radioimmunoprecipitation* played an important role in the immunochemical characterization of newly discovered antigens [7, 8]. This method is, however, extremely time consuming and too complex for the analysis of patient samples. As soon as monoclonal antibodies against platelet proteins became available, they were used for GP-specific assays as the *monoclonal*

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antibody immobilization of platelet antigens (MAIPA) [9] and *immunobeads assays* [10] Both assays can be used for detection HPA and HLA class I antibodies. The MAIPA assay as described below is an in-house assay which has gained acceptance in many laboratories. It can be used for characterization of HPA-antibodies including those against HPA-15a/b. Among commercially available test kits are *PakPlus™* (Immucor), a GP-specific ELISA, and *Pak Lx™*, an immunoassay based on the Multi-Analyte Profiling (xMAP®) technology (Luminex®). In Pak Lx, sera are incubated with a suspension of color-labeled beads carrying different platelet GPs with defined HPA antigens. Reactions are evaluated with a specialized flow cytometry instrument. Sensitivity of Pak Lx seems to be comparable to that of the MAIPA-assay [11]. Its current version it is, however, not able to detect HPA-15 antibodies and the assay is not suited for crossmatch testing.

22.3 Additional Techniques

22.3.1 Alloantibody Detection with Panels of Typed Platelets

In-house immunoassays for detection of platelet alloantibodies require that a panel of typed test platelets is available. Tests with intact platelets (e.g. platelet suspension immunofluorescence test (PSIFT)) will yield positive reactions with sera containing both HLA class I and HPA antibodies, if these platelets carry the corresponding antigens. For testing sera with PSIFT, it is very useful to have information about the specificities of HLA I antibodies. Test platelets should be obtained from donors with blood group O as platelets carry blood group A- and B-determinants.

Determination of HPA antibody specificity is simpler with GP-specific assays: Here it is useful to apply different platelet panels for each GP-complex. For GP IIb/IIIa, the panel should be able at least to identify anti-HPA-1a, -1b, -3a, -3b, (anti-HPA-4b for samples from patients of non-European descent). Panels for GP Ib/IX, Ia/IIa, CD109 should be sufficient to identify antibodies against HPA-2a, -2b, HPA-5a, -5b and HPA-15a, -15b respectively. As platelet glycoproteins carry A- and B-determinants, platelets should be obtained from blood group O donors for testing in the MAIPA assay [12].

22.3.2 Platelet Crossmatching

Testing of serum with platelets from a single selected individual is often referred to "crossmatch". Crossmatching is available with in-house platelet antibody tests and with commercial test kits.

A typical situation where platelet antibody crossmatching is useful is compatibility testing of platelet concentrates intended for use in (HLA-, HPA-) alloimmunized patients. Another situation is the completion of the diagnostic workup in cases of suspected fetal/neonatal alloimmune thrombocytopenia: if investigation of maternal serum does not yield one of the known HPA antibody specificities an alloantibody against a low-frequency antigen might be the cause. In this case a positive

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crossmatch: maternal serum tested with the father's platelets may be a sign of a maternal alloantibody against a low-frequency alloantigen [13]. In the diagnostic path for diagnosing FNAIT, a GP-specific test should be used for this crossmatch.

22.3.3 Quantitation of HPA Antibodies

Some authors suggest that the concentration of maternal anti-HPA-1a is correlated with the severity of FNAIT in case of HPA-1a-incompatible pregnancy in an immunized mother. Quantitation of the antibody is based upon a WHO international standard antibody preparation containing 100 international units (IU) per ml. The preparation is available through National Institute for Biological Standards and Control (NIBSC, U.K., code nr. 03/152). The procedure in brief: serial dilutions (1 : 2) of serum and the standard are tested in MAIPA (GP IIb/IIIa) with HPA-1a-positive platelets. Optical densities (MAIPA assay) are plotted against dilutions of serum and standard in a semilogarithmic chart. It is assumed that assays with identical optical densities contain the same concentration of specific antibody. Therefore, the concentration is derived from the distance between the sigmoid curves, ideally in their central, linear section [14, 15]. Results are expressed as I.U./ml.

22.4 Protocol: Platelet Suspension Immunofluorescence Test

22.4.1 Principle

Platelets are isolated by differential centrifugation, fixed with formaldehyde, and incubated with the serum to be investigated; after washing the platelets, they are incubated with FITC labeled anti-human IgG. After washing, the platelet suspension is evaluated by fluorescence microscope (or flow cytometry) [5].

22.4.2 Reagents

Phosphate-buffered saline (PBS): 4.7 g $Na_2HPO_4 \cdot 2H_2O$ and 8.18 g NaCl are dissolved in 900 ml of distilled water, pH is adjusted to 6.8–7.0, and distilled water is added to 1000 ml.

EDTA-PBS: 3.35 g of $Na_2EDTA \cdot 2H_2O$, 4.7 g $Na_2HPO_4 \cdot 2H_2O$ and 8.18 g of NaCl are dissolved in 900 ml of distilled water; pH is adjusted to 6.8–7.0, and distilled water is added to 1000 ml.

Formaldehyde solution: for *formaldehyde stock solution*, 4% (w/v) of paraformaldehyde is dissolved in PBS at 70°C (water bath): under gentle stirring, 1 M NaOH is added, until the solution is clear. The solution is then filtered; pH of the solution should be approximately 11. The stock solution can be stored at 4°C in the dark. *Working solution*: stock solution is diluted 1 : 4 in PBS, pH is then adjusted to 7.2–7.4.

Glycerol-PBS: 3 parts of glycerol are mixed with 1 part (v/v) of PBS solution.

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Diluted conjugate: Rabbit Anti-human IgG (Agilent Dako, Code no F0315) is diluted in PBS, the appropriate working dilution is tested for each lot of conjugate with positive sera containing platelet-reactive antibodies and negative sera without platelet reactive antibodies.

Test platelets: Whole blood anticoagulated with EDTA of donors with known HPA-antigens and blood group O is centrifuged for 5 minutes at 800 × g (or 20 minutes at 120 × g). The supernatants (platelet rich plasma (PRP)) are taken off cautiously (avoiding stirring the sedimented red cells) with a pipette, mixed (1 : 1 (v/v)) with EDTA-PBS and centrifuged in 10 ml tubes at 2800 × g for 5 minutes (room temperature). Following three washing steps (with 10 ml EDTA-PBS, centrifugation 2800 × g, 5 minutes), the platelet pellet is resuspended in 3 ml of paraformaldehyde working solution (1%) and allowed to incubate at room temperature for 2–5 minutes. Platelets are then washed twice with EDTA-PBS (10 ml EDTA-PBS, centrifugation 2800 × g, 5 minutes) and finally resuspended at a density of 150×10^3 platelets/µl. The platelet suspensions should be used within the same day.

22.4.3 Procedure

- In wells of round-bottomed microtiter plates, volumes of 50 µl of platelet suspension are incubated (30 minutes, 37°C) with 50 µl of the serum to be tested.
- Platelets are then washed three times in EDTA-PBS (100 µl, centrifugation in microtiter plate centrifuge at 1200 × g, 2 minutes).
- Platelets are incubated in 100 µl of diluted FTIC-labeled rabbit anti-human IgG, 30 minutes, room temperature.
- They are washed three times as described above and resuspended in 100 µl of glycerol-PBS.
- The platelet suspension is finally studied for platelet-bound fluorescence with a fluorescence microscope.

22.5 Protocol: Isolation of Platelets for Serological Testing

22.5.1 Principle

Blood anticoagulated with EDTA is centrifuged (low g value) and platelet rich plasma (PRP) in the supernatants is siphoned off with a pipette and transferred to a new tube. PRP is then centrifuged (higher g value). The supernatants are poured off and platelets are washed 3 times in isotonic saline. Finally, platelets are resuspended for storage in NaCl-NaN_3.

22.5.2 Reagents/Materials

NaCl-EDTA: isotonic saline with 0.5% (w/v) EDTA

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NaCl-pH6.5: isotonic saline with pH adjusted to 6.5 by adding a small volume of Dulbecco's phosphate buffered saline solution

NaCl-NaN_3: isotonic saline with 0.1% (w/v) NaN_3 (sodium azide)

Polystyrene tubes (ca 10 ml) for isolating, washing and storing platelets

22.5.3 Procedure

- Whole blood is drawn into EDTA-tubes and centrifuged in 10 ml-tubes for 20 minutes at $120 \times g$.
- The supernatants (platelet rich plasma, PRP) are taken off cautiously (avoid stirring sedimented red cells) with a pipette, mixed (1 : 1 (v/v)) with NaCl-EDTA and centrifuged in 10 ml tubes at $1600 \times g$ for 10 minutes.
- Following centrifugation, supernatants are poured off and the platelet pellet is resuspended gently in 1 ml of NaCl-pH 6.5, 9 ml of is then added and the platelet suspension is again centrifuged at $1600 \times g$.
- Following three washing steps as described above, platelets are resuspended in NaCl-NaN_3, platelet count is adjusted to approximately $200 \times 10^3/\mu l$ for storage of the platelet suspension.

22.6 Protocol: Monoclonal Antibody Immobilization of Platelet Antigens Assay (MAIPA)

22.6.1 Principle

The MAIPA assay is a glycoprotein-specific enzyme immunoassay which allows detection of HPA-alloantibodies and HLA class I antibodies, even if sera contain mixtures of antibodies. It uses monoclonal antibodies against GP IIb/IIIa, Ib/IX, Ia/IIa, CD109, and β2-microglobulin for isolation the platelet membrane protein of interest (Figure 22.1) [9, 16].

Intact test platelets are incubated in the patient's serum potentially containing platelet reactive antibodies. Platelets are washed and then incubated in buffer containing a (mouse) monoclonal platelet protein-specific antibody (mab), washed again and solubilized in buffer containing a nonionic detergent. The GP labeled with the GP-specific mab is immobilized on the plastic surface of a microtiter plate well coated with goat anti-mouse IgG. Human antibody on immobilized platelet GP is then detected with conventional ELISA techniques using enzyme-labeled goat anti-human IgG.

22.6.2 Reagents

Coating buffer: 1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, 0.2 g NaN_3 are dissolved in distilled water to 1000 ml, pH is checked and adjusted to 9.6, if necessary.

Microtiter plate coated with anti-mouse IgG: Goat anti-mouse IgG is diluted 1 : 500 in coating buffer, 100 µL of this diluted antibody solution is pipetted into wells of a flat-bottomed microtiter plate (high protein-binding capacity) and

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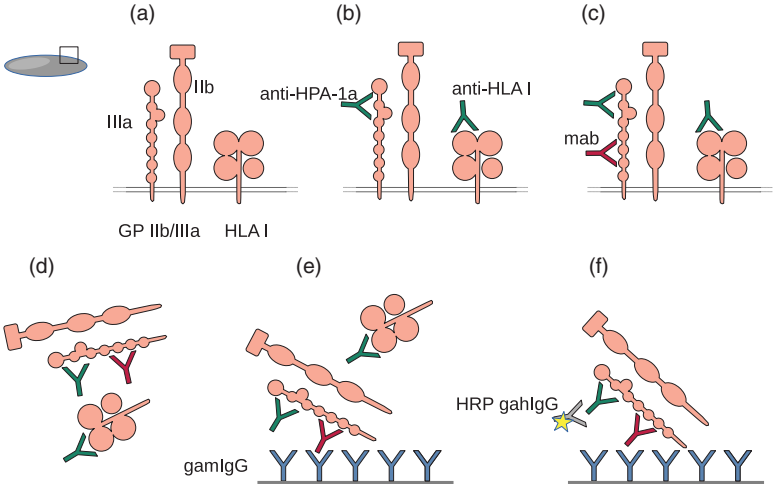

Figure 22.1 Principle of the MAIPA assay: A detail of the platelet membrane with GP IIb/IIIa and HLA class I antigen is shown (a). Intact test platelets in suspension from donors with known HPA antigens are incubated with the serum under investigation, which (in this example) contains anti-HPA-1a and HLA class I antibodies which react with GP IIb/IIIa and HLA class I antigen, respectively (b). Platelets are then washed and as only the GP IIb/IIIa-specific antibodies shall be detected in this experiment, a mouse monoclonal antibody (mab) against GP IIb/IIIa is added to the platelet suspension (c). After washing the platelets, they are solubilized in buffer containing a nonionic detergent (d). After removal of insoluble material by centrifugation, the supernatants are transferred to wells of a microtiter plate, which are coated with goat anti mouse IgG (gamIgG) (e). Only GP IIb/IIIa is immobilized through the (mouse) mab and the unbound complexes are removed by washing the microtiter plate wells (f). Anti-HPA-1a is finally detected by adding enzyme (e.g. horseradish peroxidase: HRP) labelled goat anti-human IgG (HRP gahIgG) using conventional ELISA technology.

coated at 4°C overnight. Thirty minutes before use, wells are washed four times with TBS wash buffer. Free binding sites on the plastic surface are blocked for approximately 20 minutes at 4°C with TBS wash buffer.

PBS-BSA 2%: Dulbecco's phosphate buffered saline x10 is diluted 1 : 10 in distilled water (PBS), pH is adjusted to 7.2, bovine serum albumin (BSA) is added to a concentration of 2%.

TBS wash buffer: 1.21 g Tris is dissolved 950 ml of isotonic saline, pH is adjusted to 7.4, 5 ml of Triton X-100, 0.5 ml of Tween20 and 0.5 ml of 1M $CaCl_2$ are added, isotonic saline is added to 1000 ml.

1M $CaCl_2$: 21.9 g $CaCl{\cdot}6H_2O$ are dissolved in 100 ml of distilled water.

Solubilization buffer: 1.21 g Tris is dissolved in isotonic saline, pH is adjusted to 7.4, 5 ml of Triton X-100 is added, isotonic saline is added to 1000 ml.

Substrate solution: 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution (TMB ONE™, [Kementec, Taastrup, Denmark]), this is a ready-for use-solution. The reaction is stopped with 0.18 M H_2SO_4.

PBS-BSA-EDTA: To 70 ml of Dulbecco's PBS × 10,630 ml of distilled water is added, 5 ml of BSA 30% and 10 ml of Na_2EDTA (Titriplex III); pH should be 7.2.

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Immunochemical reagents: HRP-labeled goat anti-human IgG code no 109-035-098 (Jackson ImmunoResearch), goat anti-human IgG code no 115-005-071 (Jackson ImmunoResearch).

22.6.3 Procedure

1) Test platelets are isolated from donors' EDTA-anticoagulated blood samples as described above. A total of 20×10^6 platelets suspended in approximately 50 µl of isotonic saline are centrifuged (microtiter plate centrifuge, 1200 × g, 2 minutes) in wells of a round-bottomed microtiter plate. The supernatants are flicked off and platelets are then resuspended in 30 µl of PBS-BSA 2% and 50 µl of the serum to be investigated are added.
2) Following incubation of platelets with serum at 37°C for 30 minutes, platelets are washed one time with 50 µl of isotonic saline, resuspended again in 30 µl of PBS-BSA 2% and 10 µl of the GP-specific mab (IgG concentration approximately 10–20 µg/ml) are added.
3) Platelets are incubated with the mab for 30 minutes at 37°C and washed three times with 50 µl of isotonic saline.
4) After the last centrifugation of the microtiter plate, the pellet is resuspended in 100 µL of solubilization buffer, these 100 µl are then transferred to a 1.5 mL Eppendorf reaction tube. Platelets are allowed to lyse at 4°C for 30 minutes, lysates are then centrifuged (15,000 × g for 30 minutes at 4°C) to remove particular material.
5) Following centrifugation, the upper 50 µl of the supernatants are siphoned off cautiously with a pipette and diluted in 200 µl of TBS wash buffer: 100 µl of this lysate dilution are pipetted in duplicate into wells of a flat-bottomed microtiter plate coated with goat anti-mouse IgG.
6) After incubation (90 minutes, 4 °C), the wells are tipped out and are washed four times with 200 µL TBS wash buffer, incubated with 100 µl of HRP-labeled goat anti-human IgG diluted in TBS wash buffer.
7) Finally, the microtiter wells are washed five times with 200 µl of TBS wash buffer and incubated (15 minutes in the dark, room temperature) with 100 µl of substrate solution. The enzyme reaction is stopped by adding 50 µl of 0.18 M H_2SO_4.
8) The reaction is measured with a microtiter plate photometer at 450 nm (620 nm reference wavelength).

Results are expressed as ΔO.D. (optical density) values:

$$\Delta\text{O.D.} = \text{O.D.}_{\cdot\text{test}} - \text{O.D.}_{\cdot\text{blank}}$$

($\text{O.D.}_{\cdot\text{blank}}$: "blank" value obtained with wells coated with goat anti-mouse IgG incubated with TBS wash buffer instead of diluted platelet lysate (see step no. 5))

22.6.4 Assessment of Conjugate Dilution

The concentration of HRP-labeled goat anti-human IgG conjugate is to be determined for each lot of the reagent. If the assay is set up for the first time in a

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laboratory with defined alloantibodies against antigens on GP IIb/IIIa, I/IIa, Ib/IX (and CD109) and sera without platelet reactive antibodies should be available as well as platelets with known HPA-antigens. To determine the optimal conjugate concentration, "positive assays" (sera with HPA-specific antibodies tested with antigen-positive platelets) should be run as well as negative assays (sera without platelet reactive antibodies) at different conjugate concentrations. Those conjugate dilutions should be selected, which yield rather high positive Δ O.D. values for positive reactions (serum with platelet antibody) and Δ O.D. values not higher than 0.1 for negative assays (serum without platelet antibody).

With the MAIPA assay set up, laboratories should keep a set of sera with HPA-antibodies with moderate reaction strength and sera without platelet reactive antibodies for testing new lots to ensure that sensitivity of the assay remains constant over a longer period.

22.6.5 Modifications, Comments

For testing of **antibodies against HPA-1a/b, -4b, -5a/b, and HLA class I antigens**, test platelets are isolated from EDTA anticoagulated blood by differential centrifugation and may be stored for up to some weeks at 4°C suspended in isotonic saline containing 0.1% NaN_3 as described above.

The epitopes of the HPA-2 antigens located near the N-terminal region of GP Ibα are easily cleaved off by proteases. Detection of **HPA-2a or HPA-2b specific antibodies** requires that proteases are inhibited during platelet storage and the testing procedure. In the past, this has been done by adding leupeptin (50 μg/ml) to isotonic saline during storage and to the solubilization buffer. Even more important, platelets for HPA-2 antibody testing should not be older than 3–4 days.

For detection of **anti-HPA-15a/b**, fresh platelets should be isolated one day before testing using PBS-BSA-EDTA for washing during the differential centrifugation steps. A total of 100×10^6 platelets per test is used (instead of 20×10^6 in step 1, this is required due to the low number of binding sites on platelets).

The MAIPA assay only gives positive results, if there is **no mutual inhibition of binding** of the human (HPA) antibody and the immobilizing (mouse) monoclonal antibody. This requires careful selection of mab. Table 22.2 shows the monoclonal antibody clones used for platelet alloantibody detection of the author's laboratory.

Table 22.2 Clones of capturing monoclonal antibodies used for alloantibody detection in the MAIPA assay as used in the author's laboratory.

Glycoprotein (Antigens)	Monoclonal antibody (Clone)
GP IIb/IIIa (HPA-1, -3, 4)	P2
GP Ia/IIa (HPA-5)	Gi9
GP Ib/IX (HPA-2)	FMC25
CD109 (HPA-15)	TEA 2/16
β2 microglobulin (HLA class I)	B1G6

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Other monoclonal antibody clones successfully used for HPA-antibody testing in MAIPA assay can be found in [17, 18].

In very rare cases, serum samples of patients contain antibodies yielding positive reactions with one or more GP complexes. These "*panreactive antibodies*" react with all or nearly all platelet GP from different donors. This phenomenon is sometimes observed with other GP-specific tests. In control experiments with the PSIFT, such sera yield negative results. These "panreactive" reaction patters are considered to be artifacts without clinical relevance. These artifacts must not be confused with true autoantibodies (positive reactions in PSIFT!).

22.7 New Developments for Platelet Antibody Detection

Even the widely used commercial and in-house GP-specific immunoassays have their limitations. It has been found that maternal sera in some cases with FNAIT due to HPA-1a incompatibility contained biologically active HPA-1a antibodies which were not detectable in maternal serum at the time of birth. In some of these patients, anti-HPA-1a is detectable in sera obtained some weeks later. It is assumed that such antibodies are missed due to their low affinity in currently used assays including repeated washing steps. One solution to this problem is the *surface plasmon resonance technique* [19], which detects also low-affinity HPA-1a antibodies. This method is, however; not widely available.

Other problems result from the low density of antigenic determinants on the platelet surface. This is a problem for detection of HPA-15-specific antibodies due to the low expression of CD109 on platelets. A possible solution is the generation of **bioengineered cell lines** expressing CD109 which may be used for anti-HPA-15 testing by flow cytometry or in GP-specific assays [20]. Moreover, cell lines expressing low frequency alloantigens can support laboratories in immunological testing for alloantibodies against low-frequency antigens [21].

22.8 HPA-Antigen Testing

22.8.1 Serological Testing

HPA antigens can be tested with the same immunological techniques which are used for platelet alloantibody testing. Serological antigen testing was the only choice, before the genetic basis of HPA antigens was discovered. A drawback of serological typing is the scarcity of sera with strongly reacting monospecific alloantibodies. Only anti-HPA-1a and anti-HPA-5b are widely available as typing reagents. If typing sera with HPA antibodies contain additional HLA class I antibodies, platelets have to be typed in GP-specific assays (e.g. MAIPA), if appropriate sera are available.

22.8.2 DNA-based Techniques

In 1989, the molecular basis of the HPA-1 antigen system (at that time named PlA) was described [22]. In their original publication, the authors proposed to use

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restriction enzyme analysis (Nci I) of a polymerase chain reaction (PCR) amplified product (PCR-RFLP) comprising the single nucleotide polymorphism (SNP) for routine molecular typing of patient or platelet donor samples. In short, a sequence of DNA, which comprises the polymorphic (HPA) site, is amplified by PCR. Ideally a restriction enzyme is selected, which cleaves the DNA of only one allele at the polymorphic site and at least one more cleavage site where DNA of both alleles is cleaved. This second "nonpolymorphic" site allows to control for completeness of digestion. The mixture of fragments is then subjected to agarose gel electrophoresis [23]. PCR-RFLP was extended to typing of other platelet alloantigens [24].

Nearly all platelet alloantigens result from SNPs, so rather simple assays can be used for DNA-based typing. A frequently used technique is PCR with sequence-specific primers *(PCR-SSP)*. It is based on the fact that a mismatch between primer and DNA at the 3′-nucleotide end of the primer inhibits the amplification of the DNA template. For a typing kit, a set of two primer pairs is provided with an allele-specific and a common (reverse) primer. The resulting PCR products are then analyzed by agarose gel electrophoresis. Laboratories engaged in platelet typing for diagnosis in patients often use commercial test kits based on PCR-SSP.

For testing, HPA-associated SNPs include *TaqMan™ 5′-nuclease assay* [25], *differential melting curve profile analysis (LightCycler™)* [26], and many other techniques have been described [23, 27].

A special situation for DNA typing emerges in pregnant women with a previous child suffering from fetal/neonatal alloimmune thrombocytopenia (FNAIT), usually due to anti-HPA-1a in populations of European origin. In a HPA-1(a−b+), HPA-1a immunized pregnant woman, when the father of the child is HPA-1(a+b+) it is of interest to learn, if the fetus is HPA-1(a+) or HPA-(1a−) for adequate management of the pregnancy. As an alternative to HPA-1a-typing in DNA from fetal amniotic cells (obtained by amniocentesis), fetal HPA-1a genotype may be tested in fetal DNA in maternal plasma. Different techniques have been proposed for *noninvasive fetal HPA-1a typing* [28, 29].

22.9 Concluding Remarks

Platelet antibody detection is complex and technically demanding. No single technique can detect all platelet antibodies. Therefore, technicians in experienced laboratories use more than one method. This may include in-house methods and commercial kits. Platelet alloantibody testing with in-house methods should be restricted to laboratories with sufficient proficiency. However, licensed commercial test kits for platelet crossmatch testing should be available in laboratories of hospitals, where HLA- and HLA-immunized patients receive platelet concentrates. In immunized patients receiving platelet transfusions, significant discrepancies between LCT test results and MAIPA (HLA class I) were observed [1]. The LCT is therefore not an adequate substitute for platelet antibody crossmatching in these patients.

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23

KIR Genotyping

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23.1 Complexity of *KIR* Genomic Region

The *KIR* genomic region is located on human chr19q13.4 and is comprised of 13 distinct *KIR* genes *(KIR2DL1, KIR2DL2/3, KIR2DL4, KIR2DL5A, KIR2DL5B, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5, KIR3DL1/S1, KIR3DL2, KIR3DL3*) and two pseudogenes (*KIR2DP1, KIR3DP1*) [1]. Here, "L" denotes the receptor has a long cytoplasmic tail that signals inhibition, "S" a short tail that mediates cellular activation, "P" pseudogene, and 2D or 3D denotes the number of Ig-like domains [2]. Any of the *KIR* genes may be absent from a given haplotype, some may be duplicated, and others fused in-frame to encode a functional receptor [3–7]. The centromeric portion of the KIR region spans from *KIR3DL3* to *KIR3DP1,* and the telomeric portion spans from *KIR2DL4* to *KIR3DL2* (Figure 23.1). Thus, *KIR3DL3, KIR3DP1, KIR2DL4,* and *KIR3DL2* are considered framework genes, which mark the ends of their respective portion in the majority of cases [8]. The intergenic region between *KIR3DP1* and *KIR2DL4* is highly homologous, which increases diversity of the *KIR* region through mediating recombination [1].

KIR genes are inherited in haplotypes, which can be classified into broad groups based on their gene content [9]. The *KIR-A* haplotype is defined by the presence of *KIR3DL3, KIR2DL3, KIR2DL1, KIR2DL4, KIR3DL1, KIR2DS4* and *KIR3DL2* [9]. Conversely, the *KIR-B* haplotype encompasses all other combinations of *KIR*, including a greater variety of genes encoding activating KIR (Figure 23.1). In addition to extensive gene content variability in the *KIR* region, most of the genes exhibit considerable allele diversity [10]. There are also high levels of sequence homology shared among distinct *KIR* genes. These confounding problems pose unique challenges for high-resolution genotyping using any genetic analysis method. Although a substantial number of reference sequences are available [1, 11, 12] these issues are compounded by the ongoing discovery of novel alleles and structural differences within this locus as additional populations are studied [13].

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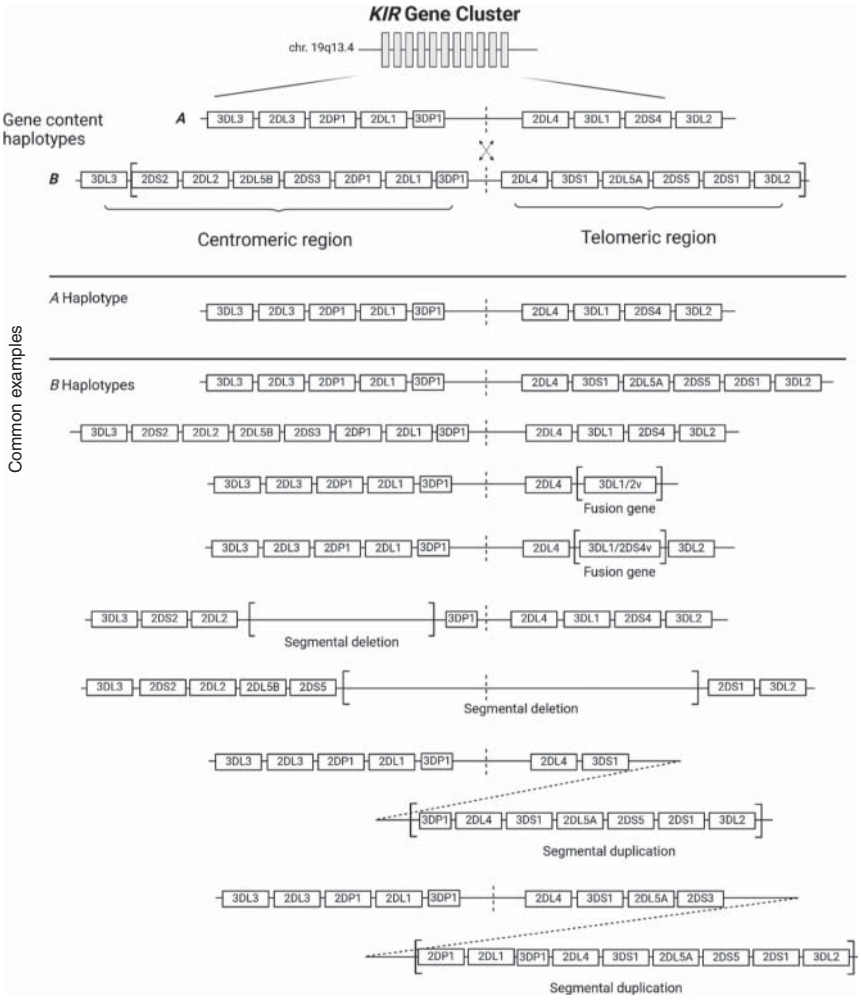

Figure 23.1 KIR haplotype model. Upper: The *KIR-A* haplotype has a fixed content of seven genes and two pseudogenes. Any other combination of KIR genes is termed a *KIR-B* haplotype. The centromeric portion of the KIR region spans from *KIR3DL3* to *KIR3DP1,* and the telomeric portion spans from *KIR2DL4* to *KIR3DL2*. (vertical dashed line) – The centromeric and telomeric portions are separated by a homologous region that mediates frequent recombination. Lower: Shows the *KIR-A* haplotype and two examples each of *B* haplotypes formed by simple recombination, gene fusion, segmental deletion or duplication.

Over the previous few decades, *KIR* genotyping methods have focused on determining the gene presence or absence variation. Several low-resolution methods exist for determining gene content, which primarily rely on PCR with sequence specific primers (SSP) or sequence-specific oligonucleotide probes (SSOP). Although these methods are relatively low throughput and unable to detect previously unseen variation, they are straightforward to incorporate in any laboratory and tend to require

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little if any specialized equipment. Recent advances in high throughput sequencing have dramatically improved the throughput and ability to fully characterize the *KIR* genomic locus [14]. This chapter will describe well-validated methods for detection of the presence/absence of *KIR* genes, *KIR* gene copy number and finally, targeted high-throughput sequencing methods for determining both gene copy and allele variation. High throughput sequencing using short read data (150–300 bp; aka NGS) is currently the state of the art for high resolution genotyping of *KIR* genes. Targeted long-read sequencing (aka third-generation sequencing) is reaching potential as a genetic test and will also be discussed.

The earliest method used to identify *KIR* gene sequences was reported by Uhrberg *et al*. in 1997 using reverse transcription polymerase chain reaction (RT-PCR) [15]. Here, peripheral blood mononuclear cells (PBMC) from 68 blood donors were compared for their expression of KIR [15]. RNA was prepared from NK clones or PBMC, and cDNA was synthesized using oligo(dt) and reverse transcriptase. PCR amplification was then performed using gene-specific primers, and the PCR products purified and subjected to direct Sanger sequencing. Using this method, the authors showed the number of expressed KIR genes varied among individuals and identified 20 distinct KIR phenotypes within this small yet ethnically diverse set of subjects. Based on these results, they developed a PCR-SSP typing system, which showed that KIR expression correlated with the presence or absence of the respective genes [15]. This method was used to establish the great extent of *KIR* gene content diversity across human populations [16]. Since then, the discovery of additional *KIR* genes [9], multiple *KIR* genotyping methods have been developed for the detection of the presence or absence of *KIR* genes, using PCR-SSP, PCR-SSOP, droplet digital PCR, or more recently sequencing of full-length *KIR* genes.

23.2 KIR Genotyping

23.2.1 The Detection of Presence and Absence of *KIR* Genes by PCR-SSP

KIR genotyping methods based on the PCR-SSP strategy have been developed by several groups [17–21]. Primers are designed to be specific for a given *KIR* gene and the reactions are amplified either individually in single tubes or multiplexed across fewer PCR reactions. Most reactions include one forward and one reverse *KIR*-gene specific primer, and in some cases additional primers are included to increase sensitivity and/or specificity of the PCR-SSP assay. Often, the reactions contain an internal positive control comprised of an additional pair of primers specific for a housekeeping gene, such as *GH1* or *HGH*. The positive control ensures that any test showing negative represents true absence of the gene and is not due to technical error. The amplicons in commonly used PCR-SSP methods range from ~200 bp to as large as ~9 kbp. As larger amplicons have greater requirement for high quality genomic DNA, amplicons of smaller length are preferred. The PCR products generated by these methods are normally visualized using an agarose

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(a) Multiplex group	KIR Gene	Amplicon (bp)	Cell line a	b	c
1	2DL1	437	+	+	+
	2DL2	164	–	+	+
	2DL4	232	+	+	+
	2DS3	279	–	+	+
2	2DL3	334	+	–	+
	2DS2	204	–	+	+
	3DL1	565	+	–	+
	3DS1	171	–	+	+
3	2DL5	113	–	+	+
	2DS1	231	–	+	+
	3DL2	142	+	+	+
4	2DS4n	130	+	–	–
	2DS4d	108	+	–	+
	2DS5	194	–	+	+
	3DL3	155	+	+	+

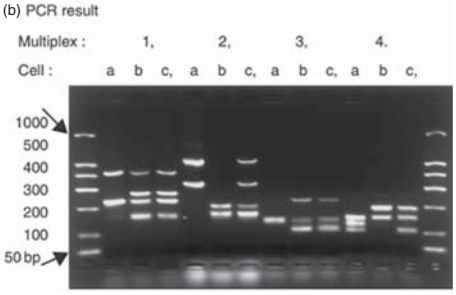

Figure 23.2 Killer-cell immunoglobulin-like receptor (KIR)-typing by multiplex PCR-SSP. Source: [20]/John Wiley & Sons. (a) A combination of *KIR* genes in each group, their amplicon length, and genotype are displayed. 2DS4n indicates *KIR2DS4* alleles with normal size and 2DS4d, alleles having a 22 bp deletion in exon 5. (b) The corresponding band of each gene is illustrated.

gel. Gomez-Lozano *et al*. (2002) described a scheme for *KIR* genotyping using 16 PCR-SSP reactions per individual [18]. In addition to the detection of the specific *KIR* genes, variants of *KIR2DL5*, *KIR2DL1*, *KIR2DL2*, *KIR2DL4* and *KIR2DS5* were amplified using additional primer sets. Sun *et al*. (2004) described the development of a multiplex PCR-SSP method for *KIR* genotyping comprised from 27 primers distributed into 14 pairs [20]. These primer pairs were used in four multiplex reactions, which together detected all *KIR* genes (Figure 23.2). They also detected a common variant of *KIR2DS4*, characterized by a 22 bp deletion in exon 5 that disables expression of the receptor [22]. Similar multiplex methods continue to be developed and refined [23, 24].

23.2.2 The Detection of *KIR* Gene Presence or Absence Using PCR-SSOP

In sequence-specific oligonucleotide probe (PCR-SSOP) methods, primers are designed to amplify broad groups of *KIR* genes and then labelled probes are used to detect specific genes or variants. Crum *et al*., described one of the earliest *KIR*-specific PCR-SSOP methods, which can define approximately 12 genes, using digoxigenin-labelled (5′) sequence-specific oligonucleotide probes [25]. The method amplified three distinct regions: (i) the transmembrane-cytoplasmic domain; (ii) the first immunoglobulin domain, and (iii) the third immunoglobulin domain. Thirteen sequence-specific oligonucleotide probes were then designed for the transmembrane cytoplasmic domain, nine probes for the first immunoglobulin domain, and four probes for the third immunoglobulin domain. The amplified DNA was bound

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to a nylon membrane, and the probes were individually hybridized according to empirically derived conditions. The specificity of all the probes and their respective hybridization conditions were verified by cloning and sequencing. This PCR-SSOP method was first used to report the *KIR* gene frequencies in 90 unrelated European blood donors and to define the *KIR* haplotypes present in 13 families [25].

The most widely used application of *KIR* genotyping by PCR-SSOP has been based on Luminex technology. In this "reverse SSOP" approach, the probes are bound to latex beads and the amplicons are labelled. Each bead has a distinct fluorescence profile, enabling determination of specificity via the sensitive dual laser detection system, similar to flow cytometry. Such *KIR* genotyping methods are often available as commercial products, such as the LABType SSO KIR kit provided by One Lambda (Thermo Fisher) [26]. This system has been used to establish the *KIR* gene presence/absence diversity of a wide range of human populations (e.g. [27]).

23.2.3 Copy Number Variation Assays

Copy number variation can be determined by PCR either using real-time or digital methods [28, 29]. Real-time PCR measures the quantity of amplicon after each round of amplification using a fluorescent readout. A typical real-time PCR amplification plot displays a sigmoidal-shaped curve (on a linear scale) and includes a baseline phase, followed by an exponential phase that reaches a plateau via a linear phase [30]. The linear phase is characterized by a linear increase in amplicon production as PCR reagents become limited and eventually exhausted. The method is quantitative when calibrated against a standard curve using data from the exponential phase of amplification. An example of a commercial RT-PCR kit for *KIR* is produced by Linkage Biosciences. Digital PCR (dPCR) can also be used for the absolute quantification of target nucleic acids. Here, the DNA sample is first partitioned into droplets where each contains either a few or no target sequences. After PCR, the fraction of amplification positive droplets is used to quantify the concentration of the target sequence, being accurately defined under Poisson distribution. Unlike real-time PCR, dPCR does not rely on calibration curves for sample quantification [30].

Jiang et al (2016) and Jayaraman et al (2019) described a real-time PCR assay termed qKAT useful for KIR genotyping and determination of copy number variation [29, 31]. qKAT consists of ten multiplex reactions performed in triplicate, each reaction including three primer pairs and three fluorescence-labeled probes that specifically amplify two *KIR* genes and one reference gene (*STAT6*). As the reference gene has a fixed copy number of two, it can be used for the relative quantification of the *KIR* gene copy number (Figure 23.3). This method utilizes primers having 3′ ends specifically matching a nucleotide that is both unique to a given KIR gene and matching all known alleles of that gene. In total, 21 primer pairs are used to detect all 13 *KIR* genes, some *KIR* gene variants and the reference gene. Amplicons range in size between 79 and 210 bp and the melting temperature of each primer is between 48 °C and 57 °C. Due to the homology across some *KIR* genes, some dual-labelled probes were designed to target exon sequences that are conserved in more than one gene. Identical probes were therefore used in different multiplex assays, which

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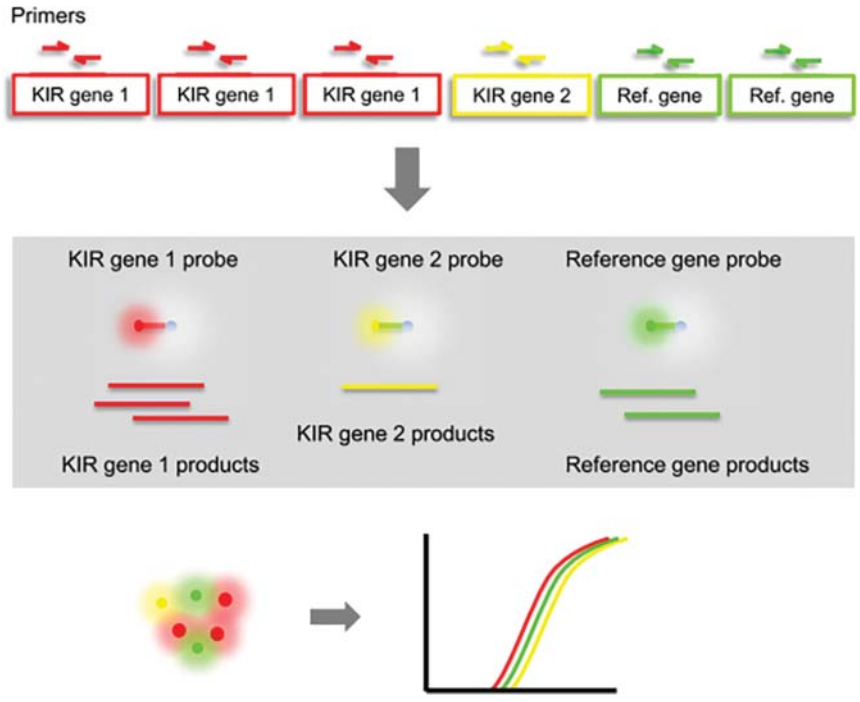

Figure 23.3 Schematic view of the KIR multiplex qPCR assay qKAT. Sequence specific primers are used for relative quantification of target KIR genes against a reference gene of fixed copy number. Each multiplex qPCR assay detects the simultaneous amplification of two target *KIR* genes and one reference gene. Two sets of primers target different exons of the two *KIR* genes, and one pair of primers amplifies the reference gene. Dual-labelled hydrolysis probes that are specific to each amplicon are used to monitor the PCR amplification in real-time. Source: [29]/Springer Nature/CC BY 4.0.

reduced the overall cost. *KIR* gene copy number is determined using comparative Cq (ΔΔCq) relative quantification analysis. This method can detect the total number of copies of each *KIR* gene with clear discrimination between 0, 1, 2, 3 or even 4 copies [29]. Like other PCR-based methods, a caveat is that newly discovered variants may not always be detected. Using *KIR*-specific oligonucleotides designed by Jiang et al (2012), Roberts *et al*. expanded this method to determine patterns of *KIR* gene linkage [32]. RT-PCR methods to measure *KIR* gene copy number continue to be developed due to their relative ease of use and accuracy [33, 34].

23.2.4 *KIR* Genotyping at the Allele Level

Whereas the PCR-SSOP approach described above provides a generic low-resolution result, it can also be used to distinguish between alleles of any *KIR* genes detected. Keaney *et al*. developed a PCR-SSOP approach specifically to determine *KIR2DL3* alleles [35]. Primers were designed to amplify the D1/D2 region, the stalk region, and the transmembrane/cytoplasmic region of the *KIR2DL3* gene. Although it was not yet established that *KIR2DL2* and *2DL3* segregate as alleles of one gene, the

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amplification of the D1/D2 region also included the amplification of *KIR2DL2*, facilitating design of oligonucleotide probes specific for the known *KIR2DL2/3* alleles [35]. Similar approaches have been used to devise allele typing methods for *KIR2DL4* [36], *KIR2DS4* [37], *KIR3DL1* and *KIR3DS1* [38, 39], *KIR3DL2* [40], and *KIR2DL2*, *KIR2DL5* and *KIR2DS5* [41].

Similarly, PCR-SSP methods have been developed that allow low-medium level resolution of the allele diversity of specific *KIR* genes. Likely the first of these was a typing scheme devised by Shilling *et al.* (2001) targeting *KIR2DL1*, *KIR2DL3*, *KIR3DL1/S1* and *KIR3DL2* [10]. This method confirmed the substantial allelic diversity of the inhibitory *KIR* genes [10]. Expanding on this protocol, Norman *et al.* (2004) mapped patterns of linkage disequilibrium and selection through the KIR locus [42], and Yawata *et al.* (2006) established the functional hierarchy amongst allotypes of KIR3DL1 [43]. Other PCR-SSP methods continue to be developed for use in disease and populations studies, and to identify further variation by virtue of unexpected reaction patterns [44–46].

23.2.5 Sequence Based Typing

Sequence based typing (SBT) using Sanger sequencing has been the gold standard for determining *KIR* allele sequences and remains the most common means for confirmation of novel alleles identified by any method [47]. Here, specific PCR primer sites are targeted to the introns flanking one or more exons of a given *KIR* gene, and the subsequent amplicons are purified to remove excess nucleotides prior to Sanger sequencing in both forward and reverse directions. Multiple methods have been developed to target individual *KIR* genes for Sanger-based SBT, including *KIR2DL4* [48], *KIR3DL3* [49], *KIR3DL1/S1* [50], *KIR3DL2* [51], *KIR2DL2/3* [52] or subsets of them [53]. Alternatively, complete methods are available targeting all *KIR* genes [54–56]. In every case, it is important to establish which *KIR* genes are present in every subject prior to attempting SBT. One SBT approach uses 2–3 amplicons per gene, with sequencing primers spaced at intervals throughout each amplicon [54, 56]. The other method generates one amplicon for every exon throughout the KIR locus [55] and is therefore less cost effective but also less reliant on the input DNA quality and simpler to optimize in the laboratory. PCR-SSP methods have also been developed to resolve any phasing ambiguities observed during SBT [56, 57].

23.2.5.1 Targeting KIR for High-Throughput Sequencing (NGS)

Two broad approaches are suitable for analyzing *KIR* genes with high-throughput sequencing. The first approach is similar to SBT, whereby specific PCR amplicons are generated and sequenced [58]. The second approach uses a panel of DNA-binding probes to isolate fragments of genomic DNA originating from the *KIR* region, which are then released from the probes and sequenced [59]. As NGS generates large numbers of short (150–300 bp) sequence reads, in both cases specialized software is required to generate the allele calls. Due to the high capacity of NGS, large numbers of individuals may be assessed on a single sequencing

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run, whereby panels of discrete adapters, often called ID tags or barcodes , enable identification post-sequencing. Vendelbosch *et al.* and Maniangou *et al.* described methods for preparing NGS libraries based on long-range PCR schemes targeting all *KIR* genes [58, 60], and van de Pasch *et al.* targeted six *KIR* genes [61], in what became a commercial product (GenDX). Although it is not possible to determine *KIR* gene copy number using these PCR-based approaches, the *KIR* allele sequences can be determined through modification of existing pipelines originally designed for HLA calling. A further PCR-based strategy was developed by Wagner *et al.* in 2018 [62]. Here, in addition to allele calling, the use of medium-length amplicons enables relative quantification of *KIR* copy number by counting the number of amplicons assigned to each gene. Although the use of the commercial kits is increasing [63, 64], Wagner *et al.* remains the most extensively used PCR-based method, virtue of its exceptionally high throughput [62].

In 2016, Norman *et al.* published a method using DNA probes to capture the *KIR* genomic region and the polymorphic *HLA* class I and II genes [59]. Modifications to the workflow were published in 2024 [65], and a similar method optimized for studies in the Japanese population was also described [66]. Genomic DNA is first fragmented using sonification or enzymatic digestion, prepared into a sequencing library and the desired fragments are harvested using biotinylated probes. Approximately 14,000 probes of 80 bp in length were designed, using the complete ImmunoPolymorphism Database (IPD [67]) as reference data [59]. Moderate binding stringency was incorporated into the probe design, permitting discovery of previously unseen variants. Using this protocol, *KIR* gene copy number is determined from the ratio of reads mapping to *KIR3DL3*, which is present at two copies in all but a handful of individuals [68]. Gene content, copy number and allele sequence genotypes are determined using purpose-built open-source software called PING (Pushing Immunogenetics to the Next Generation) [69]. A further advantage of targeting the entire *KIR* genomic region is that non-coding variations, including promoter sequences, can be analyzed [70]. Originally designed to run 96 samples using a MiSeq instrument, with the increasing capacity of short-read sequencers, the maximum number is limited by the number of ID tags that can be practically incorporated in the workflow (presently ~2,000).

23.2.5.2 Overview of DNA Library Preparation for NGS

Most methods require 100–500 ng of genomic DNA per sample. The final DNA molecules to be sequenced must be of appropriate size (600–1,000 bp) to ensure optimal instrument performance. As efficiency of data generation is dependent on fragment size, the fragments must also be of uniform size in a given instrument run. Fragmentation of genomic DNA or PCR products can be achieved through sonication or enzymatic means, with the latter being more cost effective and requiring no specialist equipment. Additionally, some enzymatic protocols can also prepare the ends of the DNA fragments for subsequent steps. These next steps are to add ID tags unique for each sample, and then the universal sequencing adapters necessary to permit the paired-end sequencing reaction to occur (Figure 23.4). Two methods for adapter ligation and sample indexing are commonly available.

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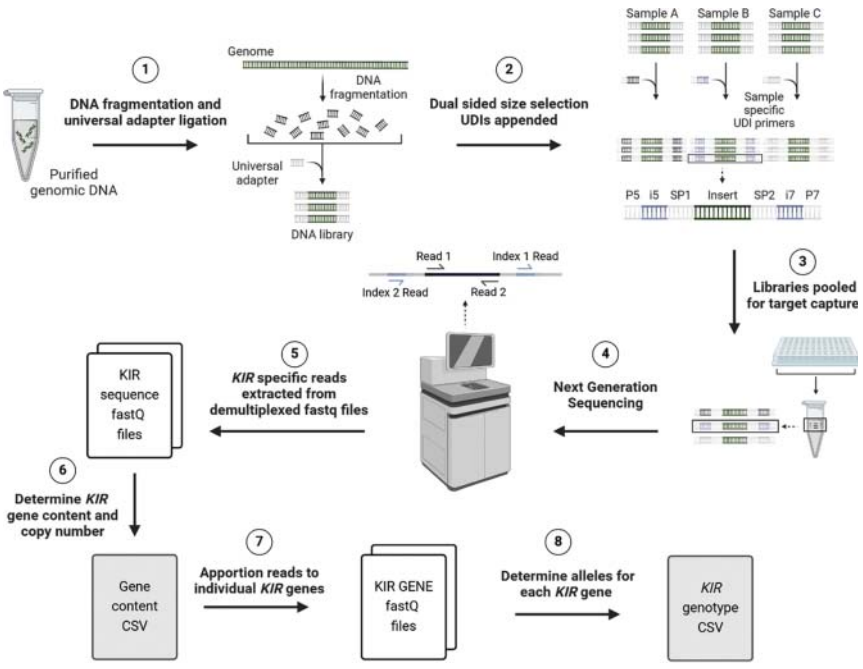

Figure 23.4 Overview of KIR genotyping using NGS [59]. (Step 1–3) Genomic DNA libraries are prepared then enriched for KIR-specific fragments using biotinylated probes. (Step 4) Enriched libraries are sequenced and (Step 5–8) resulting data is analyzed using, e.g. the PING pipeline [69]. UDI: Unique Dual Index. P5, P7; Illumina flow cell binding sequence, i7; Index 1, i5; index 2, SP1, SP2; binding sites for Illumina sequencing primers.

For lower throughput projects, including libraries to be sequenced using typical single laboratory platforms (e.g. Illumina iSeq, MiSeq or MiniSeq), combinatorial dual indexing (CDI) is often used [71]. This strategy uses 20 unique indexes, each of 8 nt, to generate 96 distinct forward/reverse combinations. Because each index may be used in multiple combinations, care must be used during downstream analysis to eliminate artifacts due to crosstalk misassignment. The second strategy, termed unique dual-indexing (UDI), uses pairs of identical 8–10 nt indexes that are unique for each sample [72]. The advantages of this approach are that it eliminates crosstalk and has greater, cost-effective, scalability, with thousands of viable unique sequences possible. Prepared libraries can then be cleaned and pooled prior to target capture or, in the case of PCR based approaches, directly prior to sequencing. The cleaning is required to remove free indexing primers, small DNA fragments, and other contaminants that will affect the sequencing quality. The most common cleaning technique uses solid-phase reversible immobilization (SPRI) beads, which can be optimized to the required fragment size by adjusting their concentration [73]. Finally, most NGS instruments employ paired-end sequencing, where, due to the homology among KIR genes, a minimum of 250 bp for each end of the fragment is recommended [65, 69].

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23.2.5.3 Downstream Analysis

For analysis of PCR products, researchers tend to adapt and validate algorithms that are concurrently used in house for HLA calling [58, 61, 62]. Others were purpose-built as part of the NGS protocol [59, 66, 69]. A common strategy of these bioinformatics pipelines is to use a universal genome sequence aligner, such as Bowtie or BWAmem [74, 75] to allocate individual sequence reads to specific *KIR* loci, and then interrogate the IPD curated sequences to determine the most likely genotypes at each locus. PING downloads the most current IPD-KIR as a reference set to ensure accurate genotype assessment and is containerized to ensure consistent performance across environments [69]. PING also uses virtual sequence probes to determine gene content diversity and solve some ambiguities directly from the raw sequence data. The method is capable of identifying up to four alleles of any duplicated *KIR* gene and has been used for a wide variety of population and disease studies [76–79]. Due to the complex and highly homologous nature of the *KIR* locus, when using probe capture with short-read NGS, genotype ambiguities may remain for some *KIR* genes at the allele calling stage. Although these issues can be solved at the population level through linkage disequilibrium, or individually through manual inspection or long-read sequencing, cost-effective high throughput long-read sequencing will ultimately become a more satisfactory alternative [14].

23.2.5.4 Determining *KIR* Genotypes from Whole Genome Studies

To leverage the vast quantity of whole genome SNP data produced over the previous few decades, Vukcevic *et al.* developed an algorithm to impute *KIR* gene presence/absence [80]. KIR*IMP can determine *KIR* gene content with 90–98% accuracy, dependent on the gene analyzed, and has been used successfully for several large disease association studies (e.g. [81, 82]). Other methods have also been developed to impute *KIR* genes [83] or alleles [84]. Whole-genome sequence data presents new opportunities and challenges for *KIR* genotyping. Broadly, a minimum of $30\times$ mean genome coverage is recommended, and care must be taken to locate all the alternative *KIR* reference haplotypes that were used in any initial genome mapping [12]. Modified versions of PING [85] as well as the T1K algorithm [86] are being developed to extract *KIR* genotypes from whole genome sequencing data, with the latter also generating *HLA* genotypes. On the other hand, two algorithms have been published to generate *KIR* genotypes from assembled, haplotype-phased genome data, as generated for the human pangenome project [87]. The structural KIR annotator (SKIRT) and Immuannot both use the current IPD-KIR as references to identify the high-resolution *KIR* genotype and identify novel variations, with SKIRT also detecting previously unseen fusion genes [7, 88]. Both these algorithms show excellent concordance with the published sequences and with each other and should become widely used with the increased prominence of phased or long-read data representing the *KIR* genomic region.

23.2.5.5 Analyzing *KIR* Using Long-read Data

As the use of long-read sequence data becomes more practical and cost-effective, the opportunities for achieving fully unambiguous *KIR* gene, allele and even haplotype

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level genotypes become realistic. The two most used long-read sequencing platforms, PacBio and Oxford Nanopore, both retain this potential through analysis of whole-genome or targeted data. Three approaches to targeting *KIR* are currently in development, long-range PCR [14], cas-9 based enrichment [89] and probe-based targeting [90]. Of these, the latter holds the greatest promise for large-scale studies due to reliance on only a small number of probes and extensive validation on an ancestrally diverse set of standards [12]. The combination of long-read data, accurate assembly, and fine-scale annotation set the perfect storm for accelerating discovery of the highly complex *KIR* genomic region.

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24

Determination of Antibodies and Typing in the HNA System

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24.1 Introduction to Granulocyte Antibody and Antigen Testing

Human neutrophil antigens (HNA) are described in Chapter 4. Whole cells are used in most assays, and therefore, it is important to note the various antigens expressed by granulocytes which include HLA class I antigens, CD15, CD45, CD16d (HNA-1), CD64, CD11a/b (HNA-4/5), CD18, CD177 (HNA-2), CTL2 (HNA-3) [1]. Cell-based assays use the isolation of granulocytes as a "target" population which fortuitously will contain mostly neutrophils. Antibody detection is based on incubating different volunteer donor-derived cells (granulocytes and lymphocytes) with previously defined HNA antigens combined with patient serum. Deducing antibody specificities from reaction patterns becomes difficult if the serum contains mixtures of granulocyte reactive antibodies. This is particularly a problem with mixtures of HLA class I and "granulocyte-specific" HNA antibodies. This is akin to the difficulties in detection of HPA specific antibodies in the presence of HLA Class I antibodies from multiparous women (see Chapter 22).

24.2 Principles of Antibody Testing

Isolation and subsequent use of granulocyte and lymphocyte populations are the beginning of most assays used in the detection of HNA antibodies**.** These assays are subdivided depending on the source of cells, either **Direct** assays using autologous patient cells or **Indirect** assays where a panel of allogeneic HNA-typed cells are utilized [2]. Direct tests have been described to be more useful in the detection of low-affinity antibodies that may fail to be detected using indirect assays especially for cases of autoimmune neutropenia.

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The most common assays used are the indirect granulocyte immunofluorescence test (GIFT) and the indirect lymphocyte immunofluorescence test (LIFT) [3–5]. The GIFT/LIFT assays involve isolation of the cell populations using density gradients and or addition of reagents to remove unwanted cells. The cell fractions are then fixed to prevent antigen internalization and are either stored frozen or used on the day of preparation. Post cell fixation, the cells are aliquoted and incubated with either HNA specific control sera or patient test serum, then washed several times, and incubated with antihuman IgG and antihuman IgM monoclonal antibodies that possess a fluorescent molecule, this is followed by another stage of wash steps, resuspension, and subsequent analysis using a flow cytometer (see Figure 24.1) [2, 3].

Each patient sample is tested against a minimum of one HNA-1a and one HNA-1b donor, but they can be tested against up to six donors to confirm an HNA specificity. The positive threshold is based on the ratio of the "mean plus four times" standard deviation of negative control sera incubated with the same cells to the patient's serum.

Interpretation of GIFT results can be complicated by cross-reactivity with HLA antibodies and the fact that some HNA molecules are expressed on cells other than neutrophils. To be able to assign an HNA specificity, the patient serum must react positively with three cells of that type and be negative for the antithetical type by GIFT only (i.e. positive with three HNA-1a cells and negative with three HNA-1b cells). If a serum is reacting with multiple cell types or this threshold is not reached, it is reported as granulocyte specific. Performing the LIFT and GIFT together aids interpretation (see Table 24.1).

The combination of GIFT and LIFT can aid interpretation, as shown in Table 24.2; reaction only in GIFT IgG suggests a lack of reactivity for HLA and HNA-3 as both antigens are expressed by lymphocytes and are thus excluded in the reaction patten provided. Table 24.2 provides an example where there is a definitive pattern of positive reactions with the GIFT assays for only cells possessing HNA-1b depicted in columns 2, 3, and 5 (Table 24.2). It is important to note that both GIFT/LIFT assays will involve both IgG and IgM antihuman conjugates to ensure both isotypes are detected.

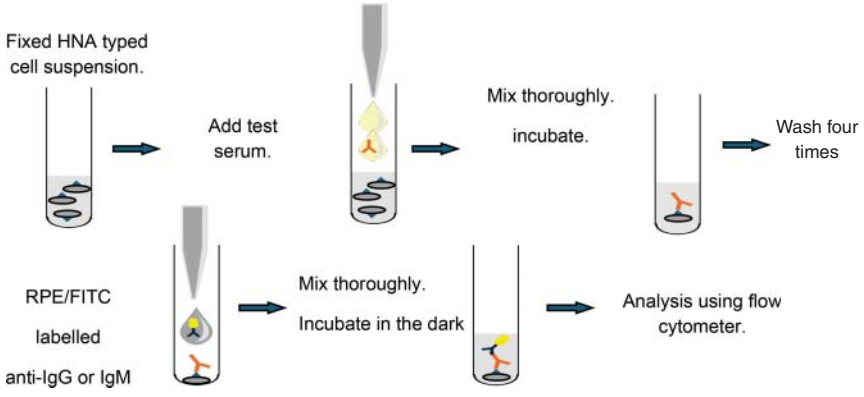

Figure 24.1 Outline of the steps involved to perform the granulocyte immunofluorescence test (GIFT) and indirect lymphocyte immunofluorescence test (LIFT).

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Table 24.1 Reaction patterns summarized for GIFT/LIFT.

Immunofluorescence test	GIFT	LIFT	Conclusion
Reaction pattern 1	+	−	Granulocyte-specific antibody
Reaction pattern 2	−	+	Lymphocyte reactive antibody/anti-HLA?
Reaction pattern 3	+	+	Lymphocyte reactive antibody±granulocyte specific? or HNA-3 antibodies?
Reaction pattern 4	−	−	Unreactive in GIFT or LIFT

Table 24.2 An example of an HNA-1b specific antibody detected using the GIFT/LIFT assays.

Donor	1	2	3	4	5
	1a, 1b, 1c, 1d **2+**, 2- **3a+**, 3b+ **4a+**, 4b+ **5a+**, 5bw+	1a, **1b**, 1c, 1**d** **2+**, 2- **3a+**, 3b+ **4a+, 4b+** **5a+,** 5bw+	1a, **1b**, 1c, **1d** **2+**, 2- **3a+,** 3b+ **4a+**, 4b+ **5a+,** 5bw+	**1a**, 1b, 1c, 1d **2+**, 2- **3a+**, 3b+ **4a+**, 4b+ **5a+, 5bw+**	1a, **1b**, 1c, **1d** **2+**, 2- **3a+,** 3b+ **4a+,** 4b+ **5a+, 5bw+**
GIFT IgG:	0.6	**7.8**	**12.0**	0.5	**6.7**
LIFT IgG	0.5	0.3	0.7	0.8	0.7

Bold font = HNA antigen positive (phenotype).
GIFT median channel fluorescence ratio = Positive >1.

In addition to GIFT and LIFT, the granulocyte agglutination test (GAT) is highly recommended [6] and even predates the use of GIFT and LIFT [5]. GAT involves isolation of HNA-typed cells usually by density centrifugation, which are then aliquoted into a microplate with a standardized cell concentration, followed by addition of patient serum and incubated at 37°C for two hours and analysis of any aggregation with an inverted microscope. GAT has been recommended for the detection of HNA-3 specific antibodies due to higher sensitivity than GIFT/LIFT [6]. A combination of GIFT/LIFT and GAT are the most widely used assays for detection of granulocyte (HNA) specific antibodies [7, 8]. Interpretation of patterns of granulocyte aggregation can be seen in Figure 24.2, where specific patterns of reactivity are shown for a HNA-specific antibody.

As mentioned previously, if HLA-Class I antibodies are present, these may mask the presence of HNA-specific antibodies, which is a major disadvantage of GIFT/LIFT assays; fortunately, a method to detect HNA-specific antibodies in the presence of HLA Class I antibodies was developed in 1993 [9], which is the monoclonal antibody-specific immobilization of granulocyte antigen (MAIGA) assay. The assay involves several steps, which requires skilled technicians to perform, see the principle of the assay in Figure 24.3.

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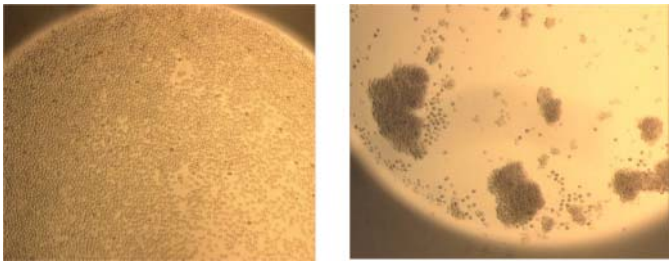

(a) Donor type = HNA-1a/1a (b) Donor type = HNA-1a/1b

Figure 24.2 HNA-1b reactivity pattern in the granulocyte agglutination test (GAT). Agglutination has occurred in the presence of HNA-1b positive cells (b), and no reaction has occurred in the absence of HNA-1b positive cells (a). Source: Photos were provided by Dr Angelika Reil, German Red Cross Blood Service, Hagen, Germany.

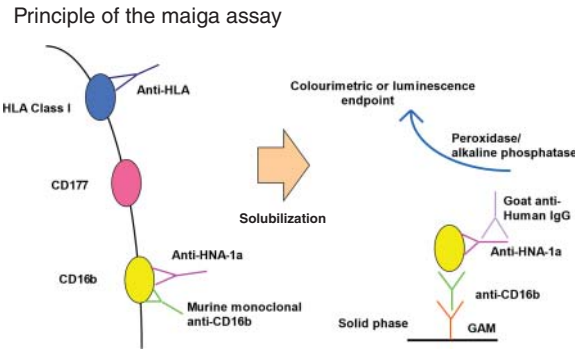

Figure 24.3 Principle of the MAIGA assay depicting the various steps involved as outlined: (a) Human neutrophil antigen (HNA)-typed donor granulocytes are added to a U-well microplate at a concentration of 2×10^7 per milliliter. (b) Patients' serum is then added to the cells, and the plate is incubated at 37°C to allow any antibodies present to bind to the HNA-typed donor granulocytes. (c) The plate wells are then washed to remove unbound antibodies. Mouse-derived antihuman monoclonal antibodies, against various glycoproteins which express the neutrophil antigens, are added and incubated at 37°C to allow them to bind to glycoproteins on the donor granulocytes, for example anti-CD16b. (d) The wells are washed to remove any unbound monoclonal antibodies. (e) Solubilization solution is added to disrupt the neutrophil membrane. The suspension is transferred to a V-well plate and incubated at 4°C for 20 minutes before it is spun in a centrifuge for 20 minutes at 4°C to sediment. (f) The suspension is transferred to an F-well flat-bottomed microplate, which has been coated with goat anti-mouse IgG. Note that the coated F-well plate should be kept at 4°C for 3 hours. (g) Blocking the plate, at least for 1 hour prior to use, will seal off any nonspecific binding sites. (h) The F-well plate is incubated at 4°C overnight to allow the mouse-derived monoclonal antibodies to bind to the goat anti-mouse capture antibodies, thus binding its specific target neutrophil antigen and any patient antibody (if present) to the F-well plate. (i) The plate wells are washed, and the plate is incubated to allow the antihuman IgG antibody to bind to any patient HNA glycoprotein antibody present. (j) The wells are washed to remove unbound antihuman IgG antibodies. A substrate is added, which reacts with the conjugated enzyme linked to the antihuman IgG antibody, to produce a color change. (k) The reaction is stopped with acid, and the optical density of each well is measured using a spectrophotometer. A positive optical density value represents the presence of patient antibodies against the specific glycoproteins. An OD value greater than 0.200 indicates a positive reaction.

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Table 24.3 Example capture antibodies utilized in MAIGA for each glycoprotein.

Glycoprotein / HNA	Antibody name
CD16 / HNA-1a, -1b and -1c	3G8
CD16 / HNA-1a, -1b and -1c	Mbc.238.7
CD16	LNK16
CD177 / HNA-2	7D8
CD177 / HNA-2	MEM166
CD11b / HNA-4	BEAR-1
CD11a / HNA-5	25.3.1
CD18 / HNA-4 and -5	7E4

The MAIGA assay depends on careful consideration on which glycoprotein-specific monoclonal antibodies are used to enable HNA-specific antibody detection, see Table 24.3. Selection on the specific capture antibody will be based on laboratory experience and may require more than one "capture" monoclonal for each antigen.

MAIGA remains the gold standard method for the identification of granulocyte (HNA)-specific antibodies, and modifications to avoid reliance on viable cells have been published, such as the use of transfected cells lines [10]. Granulocyte immunology laboratories are developing alternative assays to supplement the existing cell-based assays such as the simultaneous analysis of specific antibody assay (SASGA) [11], and recombinant HNA proteins expressed on cell lines for detection of HNA-3 antibodies [12].

Bead-based assays allow detection of antibodies to HNA-specific epitopes or even CD16b/CD177(HNA-2) glycoprotein specific antibodies. This assay can be used for detection HNA and HLA class I and class II IgG antibodies. The commercially available test kit is the LABScreen Multi (Thermofisher), a bead-based assay performed using the Multi-Analyte Profiling (xMAP®) technology (Luminex®). In the LABScreen Multi, sera are incubated with a suspension of color-labeled beads carrying different HNA-associated glycoproteins with defined HNA antigens. Reactions are evaluated with a specialized flow cytometry instrument. The sensitivity of the assay seems to be comparable to that of the cell-based assays for most HNA specificities but fails to detect some HNA-3a/b sera [13].

The use of the LABScreen Multi has proven to be a convenient method for rapid high throughput simultaneous HLA and HNA antibody screening in England (TRALI reduction intervention) [14]. The assay may also be utilized to supplement existing test algorithms but may need an IgM conjugate in addition to IgG to avoid failure to detect HNA IgM-specific antibodies.

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24.3 HNA Typing Techniques

24.3.1 HNA-Phenotyping

Most HNA typing is performed by DNA-based methods especially for HNA-1,3,4, and 5; however, due to the lack of a definitive mutation or unique single nucleotide polymorphism and the genetic complexity of the *CD177* gene [2], phenotyping remains a simple and robust approach the definition of HNA-2 status. HNA-2 phenotyping involves isolation of viable HNA-typed volunteer donor granulocytes, incubation of these cells with human anti-HNA-2 sera, and detection of bound antibodies with an antihuman IgG fluorescent conjugated antibody. There is a characteristic bi-modal expression of lower and higher levels of expression [2].

24.3.2 DNA-based Techniques

The molecular basis of the HNA system had been described, and the nomenclature publications [15–17] detail the relevant polymorphism associated with each HNA epitope (see Table 24.4). HNA-1, HNA-3, HNA-4, and HNA-5 are all based upon the presence of key single nucleotide polymorphisms (SNPs), and thus, relatively simple Polymerase Chain Reaction (PCR)-based methods have been applied including PCR-Sequence Specific Primers (PCR-SSP), PCR-Restriction Fragment Length Polymorphism (PCR-RFLP), or Real-Time-PCR (RT-PCR) methods [2, 18, 19].

Amino Acid Abbreviations

Code	Abbreviation	Amino acid name	Code	Abbreviation	Amino acid name
A	Ala	Alanine	N	Asn	Asparagine
B	Asx	Aspartic acid or Asparagine	P	Pro	Proline
C	Cys	Cysteine	Q	Gln	Glutamine
D	Asp	Aspartic acid	R	Arg	Arginine
E	Glu	Glutamic acid (Glutamate)	S	Ser	Serine
F	Phe	Phenylalanine	T	Thr	Threonine
G	Gly	Glycine	V	Val	Valine
H	His	Histidine	X		Any amino acid
I	Ile	Isoleucine	Y	Tyr	Tyrosine
K	Lys	Lysine	Z	Glx	Glutamine or Glutamate
L	Leu	Leucine	TERM Termination codon		
M	Met	Methionine			

Source: Adapted from NCBI Outreach Events.

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Table 24.4 HNA genotyping: known mutations.

Epitope	Gene	RefSeq[a)]	cDNA	SNP	Amino acid change in glycoprotein
HNA-1	*FCGR3B*	NM_000570.4[b),c)]	c.108	**C>G**	36 S>R
			c.114	**T>C**	38 L>L
			c.194	**A>G**	65 N>S
			c.233	**C>A**	78 A>D
			c.244	**A>G**	82 N>D
			c.316	**A>G**	106 I>V
HNA-3	*SLC44A2*	NM_001145056.1[d)]	c.451	**C>T**	151 L>F
			c.455	**A>G**	152 Q>R
HNA-4	*ITGAM*	NM_001145808.1[e)]	c.230	**G>A**	77 R>H[f)]
HNA-5	*ITGAL*	NM_002209.2[e)]	c.2372	**G>C**	791 R>T[g)]

a) RefSeqs from Flesch et al. [16]; and Flesch et al. [17].
b) *FCGR3B* transcript variant 2 (mature glycoprotein)
c) frequently observed SNP: NM_000570.4:c.197T>TA or T>TG (cannot exclude FCGR3A by PCR-SBT)
d) *CTL2* transcript variant 2 (expressed on neutrophils)
e) transcript variant 1
f) precursor protein (mature peptide Arg61His)
g) precursor protein (mature peptide Arg766Thr)

Sanger-based sequencing has also been developed for HNA typing [20]; the main advantage is the ability to detect novel mutations and avoid false assignment where polymorphisms may hinder primer annealing in other assays such as PCR-SSP, although this method may be less applicable to higher throughput typing [2, 20].

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25

Determination and Typing of Other Polymorphic Systems

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25.1 Introduction

This chapter guides readers through comprehensive original works that developed DNA-based methods for typing the nonclassical class-I MHC genes, *MICA/B*, *HLA-G*, *HLA-E*, and *HLA-F*. While the technical aspects of each method are briefly discussed, the primary focus is on the broader application of these methods and their variations for interrogating specific genetic *loci* based on the research question or clinical context. Updated genetic panels featuring main variants associated with specific HLA alleles are provided to assist in selecting the appropriate typing method. For those interested in detailed technical protocols, we refer to procedural guides available in specialized literature [1–5].

25.2 *MICA/B* Allele Typing Methods

The *MIC* gene family has seven members (*MICA*, *MICB*, *MICC*, *MICD*, *MICE*, *MICF*, and *MICG*) [6, 7]. *MICA* (accession No. NG_034139) and *MICB* (accession No. NG_021405) are considered functional genes while *MICC* (accession No. NG_027534), *MICD* (accession No. NG_015983), *MICE* (accession No. NG_002916), *MICF* (accession No. NG_015984), and *MICG* (accession No. NG_002733) are pseudogenes. *MICA* and *MICB* share 91% nucleotide similarity in the coding region [8], with six exons separated by five introns (Figure 25.1). *MICA* has 11.7 kb length (NCBI reference sequence NM_001177519.3) and locates ~46 kilobases (kb) centromeric to *HLA-B*. Several *MICA* variants are in linkage disequilibrium with variants in *HLA-B*, often resulting in extended haplotypes and HLA allelic associations. In transplantation settings, matching HLA-B virtually matches *MICA* alleles between unrelated donor-recipients [9]. In contrast to *MICA*, *MICB* has 12.9 kb length (NM_005931.5) and locates further at ~141 kb centromeric to *HLA-B* and does not show the same linkage disequilibrium patterns with *HLA-B*.

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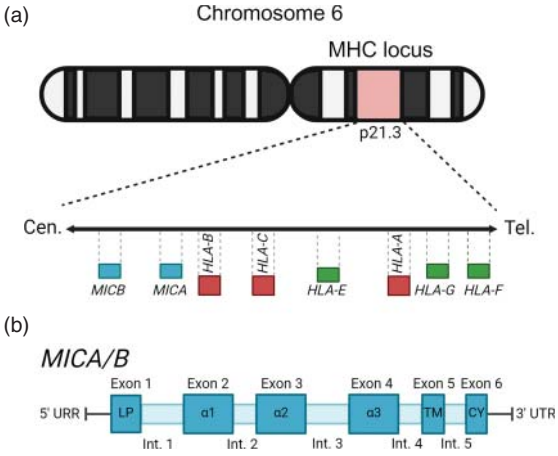

Figure 25.1 Localization, organization, and structure of the classical class I and III HLA genes within the MHC locus at p21.3. (a) schematic representation and proximity of MHC class I and III genes in the MHC locus. The classical class I HLA genes are shown in red, the non-classical class I genes in green, and the class III genes in blue. (b) schematic representation of *MICA/B* gene organization and structure. MHC, major histocompatibility complex; HLA, human leukocyte antigen; 5′URR, upstream regulatory region; 3′UTR, untranslated region; LP, leader peptide; TM, transmembrane domain; CY, cytoplasmic tail; Int, intron; Cen, centromere; Tel, telomere. The genetic distance between genes is representative and not in scale.

In *MICA/B*, Exon 1 encodes the leader peptide, exon 2 (α1 domain), exon 3 (α2 domain), and exon 4 (α3 domain) encodes the extracellular domains. Exon 5 encodes the transmembrane domain, and exon 6 is the carboxyl-terminal cytoplasmic tail followed by the 3′ untranslated region (UTR) [6] (Figure 25.1**)**. Of note, the entire length of the *MICA* cDNA sequence (~1382 bp) is shorter than *MICB* cDNA's (~2376) due to its larger 3′UTR [10].

Exons 2–4 of *MICA/B* are highly polymorphic, and a large number of alleles have been reported (IPD-IMGT/HLA report release 3.57) [11] for *MICA* (570 alleles, 281 proteins, and 9 nulls) and *MICB* (295 alleles, 49 proteins, and 25 nulls) [11]. MICA and MICB proteins have similar structures with the extracellular α domains of MHC class I genes [8], resembling their folded organization [12]. A remarkable difference is that MICA/B neither presents peptide [13] nor associates with the β2-microglobulin chain, which bridges the α1–2 domains with α3 [12]. In contrast to classical MHC-I molecules, where most variants are found in the exons 2 (α1) and 3 (α2) encoding the peptide cleft, *MICA/B* variants are found in all extracellular domains (exons 2, 3, and 4) and the intracellular domains (exons 5 and 6) [14]. In this context, several single nucleotide variants define the most frequent *MICA* (Table 25.1) and *MICB* alleles (Table 25.2) found in worldwide populations.

Methods for typing *MICA/B* have primarily focused on exons 2, 3, and 4, but it also extends to exons 5 and 6 when higher allele resolution is needed (Figure 25.2). For example, some alleles of *MICA* share identical extracellular domains but show variations in the TM region (exon 5) and, therefore, must be included in

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Table 25.1 Nonconservative and conservative amino acid changes in exons 1–6 used for typing common *MICA* alleles.

Exon/ codon	*001 (Ref)	*002	*004	*007	*008	*009	*010	*011	*012	*015	*016	*017	*018	*019	*027	*033	*045	*049
Exon 1																		
17	TTC	TTC, TTT#			TTC, TTT#													
Exon 2																		
6	CGT	CGT, CGC#					CCT											
7	TAT				TAT, TAC#							TAC, TAC#						
10	ACG				ACG, ACA#	ACG, ACA#												
11	GTG	GTG, GTA#																
12	CTG						CTG, TTG#											
13	TCC			TCC, TCT#														
14	TGG	GGG						GGG		GGG		GGG						
23	CTC		CTT		CTT, CTC#	CTT	CTT				CTT			CTT	CTT	CTT		CTT
24	ACT	GCT	GCT	GCT	GCT	GCT	GCT	GCT		GCT	GCT	GCT		GCT	GCT	GCT	GCT	GCT
26	GTA	GTA, GTG#																
34	CTG				GTG, TTG#													
36	TGT		TAT		TAT	TAT	TAT				TAT			TAT	TAT	TAT		TAT
41	TGC				TGC, TGT#													
42	AGG	AGG, AGA#																
48	CAG								CAG, CAA#									
53	GTC													GTC, GTT#				

(Continued)

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Table 25.1 (Continued)

Exon/ codon	*001 (Ref)	*002	*004	*007	*008	*009	*010	*011	*012	*015	*016	*017	*018	*019	*027	*033	*045	*049
55	GGA													GGA, GGG#				
56	AAT								AAT, AAC#									
57	ACA					ACA, ACG#												
64	AGA	AGG	AGG	AGG	AGG	AGG	AGG	AGG		AGG	AGG	AGG		AGG	AGG	AGG	AGG	AGG
67	ACA				ACA, ACC#													
69	AAC			AAC, AAT#	AAC, AAT#		AAC, AAT#											
71	AAG						AAG, AAA#											
74	AGG	AGG, AGA#																
Exon 3																		
91	CAG											CGG						
102	AAC		AAC, AAT#										AAC, AAT#					
112	TAC			TAC, TAT#		TAC, TAT#									TAC, TAT#			
114	GGG									AGG								
122	CTG		GTG			GTG												GTG
124	ACT															TCT		
125	AAG	GAG	GAG	GAG	GAG	GAG	GAG	GAG	GAG	GAG	GAG	GAG	GAG	GAG	GAG	GAG	GAG	GAG
128	ACA								ACA, ACG#									
129	ATG		GTG		GTG	GTG	GTG				GTG			GTG	GTG	GTG		GTG
136	CAG				CAG, CAA#													

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138	TTG								TTG, TTA#							
139	GCC	GCC, GCA#														
141	AAC	AAC, AAT#			AAC, AAT#	AAC, AAT#	AAC, AAT#									
142	GTC					GTC, GTT#										
143	AGG				AGG, AGA#											
150	GCC				GCC, GCG#											
151	ATG							GTG								
156	CAC								CTC							
158	CAC				CAC, CAT#	CAC, CAT#				CAC, CAT#						
164	TGC						TGC, TGT#									
165	CTG				CTG, CTT#, CTA#											
169	CGG			CGG, CGA#												
173	AAA		GAA		GAA	GAA	GAA			GAA		GAA	GAA	GAA		GAA
174	TCC				TCC, TCT#						TCC, TCT#					
175	GGC		AGC		GGC, GGG, GGT#	AGC, AGT#	AGC, AGT#		GGC, GGT#	AGC	GGC, GGG#	AGC		AGC		AGC
180	AGA		AGA, AGG#		AGA, AGG#											
181	ACA		AGA													

(Continued)

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Table 25.1 (Continued)

Exon/ codon	*001 (Ref)	*002	*004	*007	*008	*009	*010	*011	*012	*015	*016	*017	*018	*019	*027	*033	*045	*049
Exon 4																		
191	AGC	AGC, AGT#		AGT					AGC, AGT#				AGC, AGT#				AGT	
193	GCC				GCC, GCA#													
198	ATT		ATC	ATT, ATC#	ATC	ATC	ATC				ATC			ATC	ATC	ATC		ATC
205	TCT		TCC		TCC	TCC	TCC				TCC			TCC	TCC	TCC		TCC
206	GGC		AGC		AGC	AGC	AGC				AGC			AGC	AGC	AGC		AGC
210	TGG		CGG		CGG	CGG	CGG				CGG			CGG	CGG	CGG		CGG
213	ACA				ATA		ATA				ATA			ATA	ATA	ATA		
215	AGC		ACC		ACC	ACC	ACC				ACC			ACC	ACC	ACC		ACC
221	GTA				GTA, GTG#						CTA							
225	CAC		CAC, CAT#		CAC, CAT#													
229	CAG									CAG, CAA#								
247	ACC				ACC, ACT#													
251	CAA				CGA		CGA				CGA			CGA	CGA	CGA	GAA	
255	CAG								CAG, CAA#									
264	AGC				AGC, AGT#													
267	CAC	CAC, CAT#																
271	CCT							GCT										
Exon 5																		
278	CTG				CTG, CTA#													

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288	CAT				CAT, CAC#		CAT, CAC#											
294	GCT						GCT, GCG#											
295	G.CT				GGCT													
STR	GCT(4)	GCT(9)	GCT(6)	GCT(4)	GCT(5.1)	GCT(6)	GCT(5), GCT(3)#	GCT(6)	GCT(4)	GCT(9)	GCT(5)	GCT(9)	GCT(4)	GCT(5)	GCT(5)	GCT(5)	GCT(4)	GCT(6)
301	ATT				ATT, ACT#													
304	TAT	TAC								TAC		TAC						TAC, TAT
305	GTC						GTC, GTG#											
306	CGT	TGT			CGT, CAT#			TGT		TGT		TGT						
307	TGT						TGT, TGC#											
310	AAG				AAG, GAG#													
312	ACA	ACA, ACG#																
314	GCT										GCT, GCG#							
Exon 6																		
333	ACG																	ATG
350	GAT		GCT		GCT	GCT	GCT	GCT			GCT			GCT	GCT	GCT		GCT
354	ACT							GCT										
359	GGC							GGT										
360	GCC				ACC								ACC					

MICA alleles showing frequency above 1% across different populations (Klussmeier et al. 2020 [15], Zou et al. 2020 [16], Closa et al. 2021 [17], Al Hadra et al. 2022 [18]). Ref, reference allele. #, alternative codon present in rare alleles from the same allele group. STR, short tandem repeats – the (number) refers to GCT repeats. Nonconservative and conservative amino acid changes according to the IPD-IMGT/HLA Database (Release 3.57 (2024–07) Version Report – Build 6fc03c9; Barker et al. 2023 [19]).

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Table 25.2 Nonconservative and conservative amino acid changes in exons 1–6 used for typing common *MICB* alleles.

Exon/ codon	*001 (Ref)	*002	*003	*004	*005	*008	*013	*014	*018	*019	*020
Exon 1											
3	GCC	GCC, GCT#			GCC, GCT#						
13	GCC				GCC, GCT#						
Exon 2											
3	CAC				CAC, CAT#						
7	TAC							TAC, TAT#			
11	GTG	GTG, GTT#			GTG, GTT#						
12	CTG	CTG, TTG#									
16	GAA	GGA	GGA	GGA	GGA	GGA	GGA	GGA	GGA	GGA	GGA
31	CAG							CAG, CAA#			
44	AAG			AAG, AAA#	AAG, AAA#						
52	GAT			AAT							AAT
57	AAG	GAG				GAG		GAG		GAG	
66	TTG				TTG, TTA#						
70	GGG	GGG, GGA#									
78	ACT				ACT, ACC#						
Exon 3											
98	ATC					ATG					
113	GAT	AAT				AAT		AAT	AAT		AAT
158	CGC				CGC, CGT#						
Exon 4											
189	ACC		ATC								
199	ACC	ACC, ACT#									
210	CGG				CGG, CGA#		CGA				
231	GGG				GGG, GGT#						
243	ACC				ACC, ACG#						
267	CAC				CAC, CAT#						
268	GGC						AGC	AGC			
Exon 5											
277	GTG	GCG	GCG	GCG	GCG	GCG	GCG	GCG	GCG	GCG	GCG
314	GCG			GCG, GCA#	GCG, GCA#						
Exon 6											
355	GGT				GGT, GGC#						
360	GCC		ACC	ACC							ACC

MICB alleles showing frequency above 1% across different populations (Klussmeier et al. 2020 [15], Zou et al. 2020 [16], Closa et al. 2021 [17], Al Hadra et al. 2022 [18]). Ref, reference allele. #, alternative codon present in rare alleles from the same allele group. Nonconservative and conservative amino acid changes according to the IPD-IMGT/HLA Database (Release 3.57 (2024–2107) Version Report – Build 6fc03c9; Barker et al. 2023 [11]).

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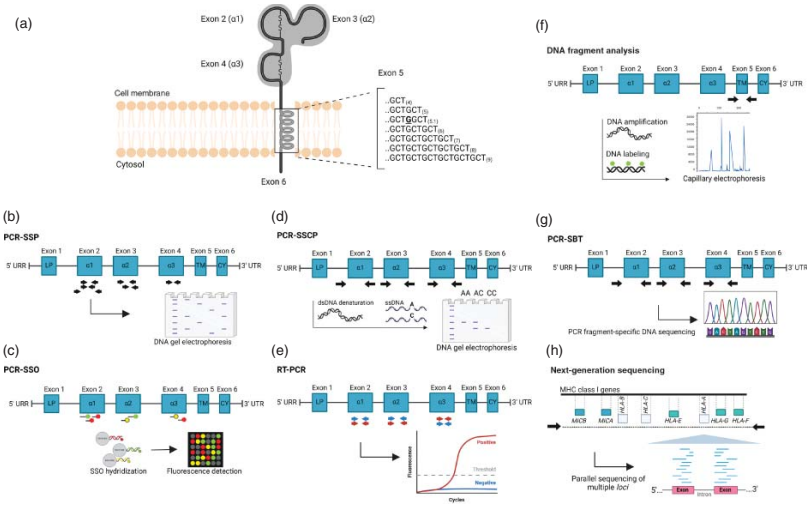

Figure 25.2 Schematic representation of methods developed for *MICA/B* typing. (a) Representation of mature MICA (MHC class I polypeptide-related sequence A) protein expressed on the cell membrane. The polymorphic exons 2, 3, and 4 encode the extracellular domains, exon 5 encodes the transmembrane domain where GCT repeats affect the length of the cytoplasmic tail. The schematics represent different DNA-based approaches and informative genomic regions used for *MICA/B* typing: (b) PCR-SSP, sequence-specific primer; (c) PCR-SSO, sequence-specific oligonucleotide probes; (d) PCR-SSCP, single-strand conformation polymorphism; (e) RT-PCR, real-time PCR; (f) DNA fragment analysis; (g) PCR-SBT, sequencing-based typing; and (h**)** NGS, next-generation sequencing. Solid arrows of opposing direction represent PCR-based amplifications and informative genomic regions for *MICA/B* typing.

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typing methods to avoid typing ambiguities [20]. Traditional and current typing methods include low- and high-resolution techniques (Table 25.3). PCR-SSCP [single-strand conformation polymorphism or PCR-DCP (DNA conformation polymorphism)], PCR-SSOP (sequence-specific oligonucleotides probes), PCR-MSNE (multiplexed single nucleotide extension), PCR-SSP (sequence-specific primer), SBT (sequencing-based typing), and NGS (next-generation sequencing) have been used in determining *MICA/B* allelic diversity (Table 25.3).

PCR-SSCP [also known as PCR-DNA conformation polymorphism (DCP)] requires PCR amplification of a genomic region of interest, denaturation of dsDNA fragment with heat and denaturants (e.g. formamide, sodium hydroxide, urea, and methylmercury hydroxide) followed by non-denaturing polyacrylamide gel electrophoresis [53]. Genotyping is based on banding patterns (electrophoretic mobility) of the ssDNA, which is sequence-dependent (i.e. mobility is affected due to the presence of single nucleotide variants). PCR-SSCP has been developed to characterize *MICA* allele diversity. A set of three primers targeting variants in *MICA* exons 2, 3, and 4 has been used to identify 16 *MICA* alleles segregating in a Japanese population [21, 24]. Despite being considered a rapid, inexpensive, and convenient method, multiple conformations of *MICA* ssDNA due to DNA polymerase error rate and sequence-dependent folding or new variants complicate interpreting banding patterns, requiring previous knowledge of allele sequences.

PCR-SSOP has been developed for *MICA/B* typing and validated using comprehensive sets of established cell lines (International Histocompatibility Workshop cell lines panel) [26, 37–39]. In this context, *MICA* typing is accomplished using a few SSOPs targeting exons 3 and 4 [37]. However, more comprehensive coverage is necessary when working with human populations due to increased genetic diversity in the *MICA* locus [26, 38]. While both PCR-SSCP and PCR-SSOP require prior knowledge of allele sequences, unusual banding and hybridization patterns are helpful in identifying new alleles when combined with DNA sequencing.

PCR-MSNE is a method based on DNA amplification, hybridization, and fluorescence analysis developed for typing *MICA* [36]. The method incorporates a single labeled ddNTP (dideoxynucleotide) complementary to the polymorphic site. The single base extension reaction in the PCR template is guided by a complementary tagged-oligonucleotide probe/primer specific for the polymorphic site. The resulting PCR products are hybridized with polystyrene microspheres (i.e. "beads") containing unique anti-tag sequences and subject to flow cytometry-based analysis. Genotype determination is assigned based on fluorescence intensity [54].

PCR reverse SSO (rSSO) is a variation of PCR-MSNE (sequence-specific oligonucleotide) and has been commercially developed for *MICA* allele typing (One Lambda, USA). Briefly, allele-specific probes are immobilized in labeled beads in microwell plates, and DNA amplified by biotinylated primers is hybridized with probes coupled to beads. After hybridization, heteroduplexes are labeled with R-phycoerythrin–conjugated streptavidin (SAPE), and *MICA* allele typing is based on fluorescence.

PCR-SSP ("phototyping") has been used for *MICA/B* typing. Most methods target variants in exons 2–4 [24–27], with some extending it to exons 5–6 [29]. Of note,

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Table 25.3 DNA-based methods developed for *MICA/B* typing.

Method	Target regions for *MICA/B* typing	References
PCR-SSCP		
	MICA exons 2, 3, and 4	Tsuchiya et al. 1998 [21]
	MICA exon 5 (STR)	Kimura et al. 1999 [22]
	MICB exon 4	Fischer et al. 1999 [23]
PCR-SSP		
	MICA exon 2	Komatsu-Wakui et al. 1999 [24]
	MICA exons 2, 3, and 4	Stephens et al. 1999 [25]
		Obuchi et al. 2001 [26]
		Marin et al. 2004 [27]
		Jumnainsong et al. 2007 [28]
	MICA exons 2, 3, 4, and gene deletion	Collins et al. 2002 [29]
	MICA exons 2, 3, 4, 5, 6	Ahmad et al. 2002 [30]
		Rees et al. 2005 [31]
	MICB exons 2, 3, 4	Collins et al. 2002 [29]
		González et al. 2003 [14]
	MICB exons 2, 3, 4, 5, 6	Ahmad et al. 2002 [30]
		Rees et al. 2005 [31]
PCR-fragment analysis		
	MICA exon 5 (STR)	Mizuki et al. 1997 [32]
		Fodil et al. 1996 [33]
		Ahmad et al. 2002 [30]
		González et al. 2003 [14]
		Katsuyama et al. 1999 [34]
	MICB intron 1 (STR)	Kimura et al. 1998 [35]
PCR-MSNE		
	MICA exons 2, 3, and 4	Zhang et al. 2003 [36]
PCR-SSOP		
	MICA exons 2, 3, and 4	Mendoza-Rincon et al. 1999 [37]
		Zhang et al. 2001 [38]
		Obuchi et al. 2001 [26]
	MICB exons 2, 3, 4, and 5. Introns 2 and 4	Fischer et al. 2000 [39]
PCR-rSSO		
	*MICA**001, *002, *007-*012, *017, *019, *033, *045	Edinur et al. 2013 [40] Zou et al. 2020 [16]

(*continued*)

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Table 25.3 (Continued)

Method	Target regions for *MICA/B* typing	References
PCR-SBT		
	MICA deletion	Komatsu-Wakui et al. 1999 [24]
	MICA exons 2, 3, and 4	Fodil et al. 1996 [33]
		Katsuyama et al. 1999 [34]
		Petersdorf et al. 1999 [41]
		Yao et al. 1999 [42]
	MICA exons 2, 3, 4, and 5	Visser et al. 1999 [43]
		Zou et al. 2006 [20]
		Zou et al. 2020 [16]
	MICA exons 2, 3, 4, 5, and 6. Introns 2, 3, and 4	Obuchi et al. 2001 [26]
	MICA exon 5 (STR)	Mizuki et al. 1997 [32]
	MICA from exons 2 to 5	Romphruk et al. 2001 [44]
	MICB exons 2, 3, 4, and 5	Ando et al. 1997 [45]
		Pellet et al. 1997 [46]
	MICB exons 1–6 (cDNA)	Visser et al. 1998 [47]
	MICB exons 2, 3, and 4	Cha et al. 2011 [48]
	MICB exons 2, 3, 4, 5, and 6	Ying et al. 2013 [49]
NGS[a)]		
	MICA 5'untranslated region (UTR) to the 3′UTR	Zhou et al. 2016 [50]
		Ivanova et al. 2024 [51]
		Zou et al. 2020 [16]
		Closa et al. 2021 [17]
		Al Hadra et al. 2022 [18]
		Ormandjieva et al. 2023 [52]
	MICA exons 2, 3, 4 and 5	Klussmeier et al. 2020 [15]

PCR, polymerase chain reaction; PCR-SSCP, single-strand conformation polymorphism; PCR-SSP, sequence-specific primer; PCR-MSNE, multiplexed single nucleotide extension; PCR-SSOP, sequence-specific oligonucleotides probes; PCR-SBT, sequencing-based typing; NGS, next-generation sequencing; cDNA, complementary DNA; STR, short tandem repeat.

a) NGS (next generation sequencing) platforms, Zou et al. 2020 = Illumina MiSeq and Oxford Nanopore Technology MinION. Ivanova et al. 2024 = Oxford Nanopore Technology. Closa et al. 2021 = Illumina MiSeq. Al Hadra et al. 2022 = unavailable. Zhou et al. 2016 = Illumina HiSeq2000. Ormandjieva et al. 2023 = Illumina MiniSeq. Klussmeier et al. 2020 = Illumina HiSeq2500.

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specific primer combinations in PCR-SSP can detect *MICA* gene deletion [24]. Most methods have been tested and validated using known HLA-typing cells (International Histocompatibility Workshop cell lines panel) and unrelated individuals. The quantity of sequence-specific primer pairs has increased over time, ranging from 20 [25] to 76 [27], to detect new alleles and to account for populational diversity. In this context, similar nucleotide composition of alleles requires additional primer sets to discriminate homozygous from heterozygous individuals. For example, some *MICA* alleles differ only by the microsatellite polymorphism in exon 5 [30]. When designing primers for *MICA* and *MICB,* the high sequence homology in their upstream regions (telomeric) should be avoided due to nonspecific amplifications; this is particularly important when typing *MICA* deletion [24, 55].

Low-resolution techniques, such as PCR-SSP, PCR-SSOP, and MSNE, have been used to generate populational estimates of *MICA* allele frequencies in healthy unrelated individuals, such as Brazilians [27], Japanese [24, 26], and Americans of African ancestry [36] and North Americans [38] when higher allelic resolution is not required.

SBT has been used to verify new alleles identified by low-resolution techniques (e.g. unusual hybridization pattern in PCR-SSOP or banding in PCR-SSCP) and to play a significant role in characterizing *MICA/B* allele diversity. Traditionally, *MICA/B* sequencing was performed on either PCR-amplified exons 2–4 [8] using generic primers [33] or by direct sequencing of individual amplified exons [26, 33, 41, 43] using custom primers. Similarly, *MICB* sequencing has been performed on direct sequencing of PCR-amplified exons 2–6 [49] or individual exons 2, 3, 4, and 5 [45, 47]. Of note, the inclusion of *MICB* exon 6 (cytoplasmic tail) discriminates previous ambiguous typing of *MICB* alleles due to the presence of a single variant [49].

NGS yields high-throughput *HLA* and *MICA/B* typing and has been included in genotyping workflow in clinical settings [15] and population-based investigations [17, 18]. The method has been used to type thousands of human samples and provides significant estimates of *MICA/B* allelic frequencies in Europeans [15, 17, 18] and a Chinese [16] population. Sequencing strategies vary; by sequencing exons 2, 3, 4, and 5, a high resolution can be achieved by identifying the most frequent *MICA/B* alleles [15]. As a limitation of this strategy, allele ambiguities may occur due to missing sequence information (e.g. noninclusion of exon 6) and phasing ambiguities (i.e. phasing is uncertain because short amplicons do not overlap, resulting in erroneous allele estimates). In this context, up to 3% and 24% of phasing ambiguities are reported for *MICA* and *MICB*, respectively [15].

In contrast to other HLAs, most *MICA/B* phasing ambiguities can be solved statistically since the combination of two rare alleles would be highly unlikely if the alternative option included two common alleles [15]. An alternative approach for addressing ambiguities mentioned above in *MICA* typing or confirming new alleles is based on using multiple NGS platforms [16, 51]. By leveraging different sequencing technologies to overcome NGS platform limitations (e.g. Illumina's high accuracy of base calling and short reads and Oxford nanopore technology's long reads

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and lower base calling accuracy), a high accuracy has been reached in determining *MICA* allele sequences and phasing reducing previously observed allele ambiguities.

Several methods for typing *MICA/B* have been developed since its discovery in 1994 [8, 56]. These methods include many PCR-based techniques and next-generation sequencing, each offering advantages and limitations. As emerging data highlight the involvement of *MICA/B* in various diseases [57] and transplantation outcomes [58], the choice of *MICA*-typing method depends on the study design and translational applicability. High-resolution methods, while more time-consuming and expensive, provide greater detail and are essential for precise genetic matching in transplantation and for detailed population studies. However, genetic association studies may not require a high level of allelic resolution. Therefore, selecting the appropriate MICA typing method requires a careful balance of these factors to ensure reliable and relevant results.

25.3 *MICA/B* Single Nucleotide Variant Genotyping Methods

Apart from *MICA/B* allele typing, single nucleotide variants (SNVs) with functional impact have also been investigated. SBT has been used to investigate the short tandem repeat (STR) of trinucleotides (GCT/Ala) repeats in *MICA* exon 5 [22, 32]. The number of triplet repeats defines transmembrane alleles: A4 (*MICA*-1.4), A5 (*MICA*-1.3), A6 (*MICA*-1.2), A7, A8, A9 (*MICA*-1.1), and A10. A variation of the A5 allele, A5.1, contains five GCT repeats plus an additional guanine nucleotide insertion (GGCT). This insertion results in a premature stop codon within the transmembrane region, resulting in the absence of MICA cytoplasmic tail [32] and increased soluble MICA levels [59, 60]. Of note, the most frequent MICA*008 has the A5.1 allele and lacks its transmembrane domain.

PCR-RFLP (restriction fragment length polymorphism) [61] and real-time PCR [62] have been developed for genotyping the *MICA* 129Val/Met dimorphism caused by an SNV (rs1051792) at nucleotide position 454 (G>A) in exon 3 (α2 domain). This variant classifies *MICA* alleles into NKG2D-receptor low (Val)- and high (Met)-affinity binding alleles [63]. Furthermore, matching *MICA*, including the *MICA*-129 dimorphism, in transplantation settings has been associated with improved outcomes [9, 62, 64, 65]. Finally, a DNA fragment analysis method was developed to genotype another STR in *MIC-B* located in intron 1 with unknown biological significance. The variant consists of several CA/TG repeat units [32]; each repeat defines CA-13 to CA-28 alleles. Another STR in intron 1 with unknown biological impact has been identified by PCR-SSP, the *AluY* (Alu subfamily Y) repeat dimorphism [66, 67]. Further, SBT has been used to identify *MICB* null alleles caused by variants at nucleotide positions 135 and 170 of exon 2, leading to premature stop codons [45, 68].

25.4 *HLA-G* Allele Typing Methods

The *HLA-G* is located on chromosome 6 and is telomeric to *HLA-A* (ca. 119 kb) and centromeric to *HLA-F* (ca. 107 kb). Interestingly, *HLA-G* shows linkage

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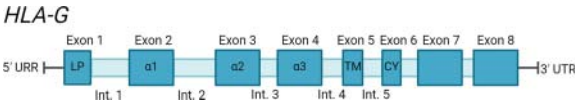

Figure 25.3 . **Schematic representation of *HLA-G* gene organization and structure.** The gene has eight exons separated by seven introns. Exons 7 and 8 are not translated due to a premature stop codon in exon 6. Therefore, this gene segment has been considered the 3′UTR. 5′URR, upstream regulatory region. 3′UTR, untranslated region; LP, leader peptide; TM, transmembrane domain; CY, cytoplasmic tail; Int, intron.

disequilibrium with *HLA-A, HLA-F*, and *HLA-H* alleles [69, 70], and a specific *HLA-G* allele is also found in linkage disequilibrium with HLA-E in an isolated population [71] despite rare. Of note, LD patterns are population-specific as no association was found in admixed populations between *HLA-G* and other HLA class I and II alleles [72].

According to the NCBI reference sequence, *HLA-G* (NM_001384290.1) length is 3197 bp, and its exon/intron organization is similar to *HLA-A*, *-B*, and *-C* genes (Figure 25.3). Exon 1 encodes the peptide signal, and exon 2, exon 3, and exon 4 encodes the α1, α2, and α3 extracellular domains, respectively. Exon 5 encodes the transmembrane domain, and exon 6 encodes the cytoplasmic tail. Exons 7 and 8 are not translated due to a premature stop codon in exon 6. Therefore, this gene segment has been considered the 3′ untranslated region [73]. Despite the high amino acid sequence identity (~80%) of HLA-G and other HLA class I proteins [74], HLA-G is a 39 kDa protein (6 kDa smaller than HLA-I). Also, it has no endocytic motif conferring a prolonged half-life on the cell membrane compared to its counterparts [75].

HLA-G is one of the least polymorphic genes among MHC-Ib. In total, 169 alleles, 51 proteins, and 6 nulls were reported (IPD-IMGT/HLA report release 3.57) [11]. Due to *HLA-G*'s low polymorphism in the coding region, methods for allele typing have primarily focused on sequence variations in exon 2 (α1 domain) and exon 3 (α2 domain) and a few variants in exon 4 (α3 domain). Table 25.4 describes the most frequent worldwide *HLA-G* alleles and the polymorphic sites used for allele typing.

Since Geraghty and colleagues [77] cloned *HLA-G*, a comprehensive number of DNA-based typing methods have been developed for *HLA-G*, such as PCR-RFLP, PCR-SSCP, PCR-SSOP, PCR-SSP, DNA fragment analysis-based genotyping, PCR-SBT and NGS (Table 25.5).

Because SNVs in exons 2, 3, and 4 have complementary sequences for specific restriction endonuclease enzymes (i.e. *HinfI*, *MspI*, *AcyI*, *DraII,* and *Eco72I*) and produce predictable cleavage patterns, DNA amplification of the abovementioned exons followed by restriction endonuclease digestion (PCR-RFLP) are used for determining variants in codon 31 (*HinfI;* Thr→Ser), 57 (*MspI*; Pro→Pro), 107 (*AcyI*; Gly→Gly), 130 (*DraII or Sau96I*; Leu→frameshift), 258 (*Eco72I*; Thr→Met), and others [78–84, 91]. Of note, the use of alternative restriction endonucleases is possible due to the existence of enzyme isoschizomers, which are enzymes that recognize identical DNA sequences (e.g. *MspI: BsiSI, HapII, AcyI: BsaHI, BssNI, BstACI, Hin1I, Hsp92I, DraIII: AdeI, Eco721: AcvI, BbrPI, PmaCI, PmlI,* and *PspCI)*.

Despite the limited typing resolution of PCR-SSCP, its use in earlier investigations on *HLA-G* diversity was fundamental in identifying several alleles, including the

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Table 25.4 Nonconservative and conservative amino acid changes in exons 2–5 used for typing common *HLA-G* alleles.

***HLA-G* Allele**	**EXON 2**			**EXON3**				**EXON 4**			**EXON 5**		**Frequency[a)]**
Codon	31	57	69	93	107	110	130	188	258	267	290	309	
G*01:01:01 (Ref)	ACG	CCG	GCC	CAC	GGA	CTC	CTG	CAC	ACG	CCG	GGC	AGA	0.385
G*01:01:02		CCA		CAT							GGT	AGG	0.153
G*01:01:03		CCA			GGT							AGG	0.074
G*01:01:22		CCA		CAT								AGG	0.013
G*01:03:01	TCG							CAT				AGG	0.057
G*01:04:01		CCA				ATC						AGG	0.132
G*01:04:04		CCA				ATC				CCA		AGG	0.053
G*01:05:01N		CCA		CAT			–TG				GGT	AGG	0.024
G*01:06:01		CCA		CAT					ATG		GGT	AGG	0.049

HLA-G alleles showing frequency above 1%.

a) Estimated *HLA-G* allele frequency in 4640 samples from across the world (Castelli et al. 2021 [76]). Ref, reference allele. –, nucleotide deletion. Nonconservative and conservative amino acid changes according to the IPD-IMGT/HLA Database (Release 3.57 (2024–2107) Version Report – Build 6fc03c9; Barker et al. 2023 [11]).

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Table 25.5 DNA-based methods developed for *HLA-G* typing.

Method	Target regions for *HLA-G* typing	References
PCR-RFLP		
	Exons 2 and 3	Morales et al. 1993 [78]
		Ober et al. 1998 [79]
		Hviid et al. 1998 [80]
		Gómez-Casado et al. 1999 [81]
	Exon 3	Hviid et al. 1997 [82]
		Lin et al. 2009 [83]
	Exon 3 cDNA	Hviid et al. 1998 [80]
	Exon 4	Hviid et al. 2002 [84]
PCR-SSCP		
	Exons 2, 3, 4, 5, and 6	van der Ven and Ober 1994 [85]
	Exons 2, 3, and 4	Yamashita et al. 1996 [86]
	Exons 2, 3, 4, and 5	van der Ven et al. 1998 [87]
	Exons 2, 3, and intron 2	Suárez et al. 1997 [88]
	Exon 3	Hviid et al. 1997 [82]
	Exon 3: genomic and *HLA-G* cDNA	Hviid et al. 1998 [80]
PCR-SSP		
	Exon 3	Hviid et al. 1998 [80]
	Exons, 2, 3, 4	Moreau et al. 2008 [89]
ARMS-PCR		
	Exons 2 and 3	Matte et al. 2000 [90]
PCR-SSOP		
	Exon 3	Yamashita et al. 1996 [86]
	Exon 2 to exon 3	Ober et al. 1998 [79]
		Alvarez et al. 1999 [91]
		Gómez-Casado et al. 1999 [81]
		Aldrich et al. 2001 [92]
PCR-fragment analysis		
	Exon 3	Bermingham et al. 2000 [93]
	Exons 2, 3, 4, and 8	Di Cristofaro et al. 2011 [71]
	HLA-G cDNA	Hviid et al. 1998 [80]
PCR-SBT		
	HLA-G cDNA (cDNA)	Ishitani and Geraghty 1992 [94]
		Fuji et al. 1994 [95]
		Kirszenbaum et al. 1994 [96]
		Hviid et al. 1998 [80]
		Román et al. 2009 [97]

(*continued*)

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Table 25.5 (Continued)

Method	Target regions for *HLA-G* typing	References
	Exons 2, 3, 4, 5, and 6	van der Ven and Ober 1994 [85]
	Exon 2, 3, and 4	Yamashita et al. 1996 [86]
		Castelli et al. 2007 [98]
		Turk et al. 2008 [99]
		Castelli et al. 2010 [100]
		Ferguson et al. 2011 [101]
		Arnaiz-Villena et al. 2016 [102]
		Wang and Tian, 2016 [103]
		Castro et al. 2019 [72]
		Wang et al. 2016 [104]
	Exon 2 and 3	Ober et al. 1996 [69]
		Hviid et al. 2002 [84]
		van der Ven et al. 1998 [87]
		Gómez-Casado et al. 1999 [81]
	Exon 3	Hviid et al. 1997 [82]
	5′URR	Ober et al. 2003 [105]
		Tan et al. 2005 [106]
	3′UTR	Castelli et al. 2010 [100]
		Wang et al. 2016 [104]
		Craenmehr et al. 2019 [107]
	Full gene	Pyo et al. 2006 [108]
	Exons 1, 2, 3, 4, and 5	Stieglitz et al. 2019 [109]
NGS[a)]		
		Castelli et al. 2017 [110]
		De Oliveira et al. 2018 [111]
		Ribeyre et al. 2018 [112]
		Nilsson et al. 2018 [113]
		Nishizawa et al. 2020 [114]
		Alizadeh et al. 2020 [115]
		Drabbels et al. 2020 [116]
		Williams et al. 2021 [117]
		Ormandjieva et al. 2023 [52]

PCR, polymerase chain reaction; PCR-RFLP, restriction fragment length polymorphism; PCR-SSCP, single-strand conformation polymorphism; PCR-SSP, sequence-specific primer; ARMS-PCR, Amplification-refractory mutation system PCR; PCR-SSOP, sequence-specific oligonucleotides probes; PCR-SBT, sequencing-based typing; NGS, next-generation sequencing; cDNA, complementary DNA; 5URR, upstream regulatory region; 3UTR, untranslated region.

a) NGS platforms, Castelli et al. 2017, De Oliveira et al. 2018, Ribeyre et al. 2018: Illumina MiSeq. Nilsson et al. 2018: Thermo Fisher Scientific, Ion Torrent. Nishizawa et al. 2020: PacBio single-molecule real-time sequencing. Alizadeh et al. 2020: Illumina MiSeQ. Drabbels et al. 2021: Illumina. Williams et al. 2021: Illumina. Ormandjieva et al. 2023: Illumina MiSeq.

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G*01:01:05N null allele and others, which was confirmed by either cloning or direct DNA-fragment sequencing [79, 80, 82, 85–88]. Further, an alternative approach of PCR-SSCP consists of using *HLA-G* cDNA as a source for amplification instead of genomic DNA for interrogating or validating the presence of amino acid changes in exon 3 [80].

Among the methods used to type *HLA*-G, PCR-SSP techniques focused mainly on determining non-synonymous (conservative) amino acid changes defining *HLA-G* alleles, such as G*01:01 to G*0107 [89]. Variations in the technique also target single synonymous amino acid changes, such as the codon 93 (His→His) [82]. Interestingly, ARMS (Amplification Refractory Mutation System)-PCR was developed to genotype variants in codons 93 and 110 [90]. The technique uses a single PCR reaction with four primers, two primers complementary to the polymorphic site in the 3′ end (3′C or 3′T) designed in opposite directions. The other primer pair amplifies the region surrounding the polymorphic site, and genotypes are determined based on PCR fragment length detected in gel electrophoresis.

PCR-SSO probes were also designed to type the most frequent *HLA-G* alleles targeting synonymous and non-synonymous variants found across exons 2–4 [79, 81, 86, 91, 92]. Notably, previous PCR-SSO protocols do not differentiate other G*01:01 (G*01:01:02, G*01:01:03, and G*01:01:04) and G*01:04 (G*01:04:04) alleles. However, the inclusion of additional four SSO probes targeting codons 188 (His→His), 258 (Thr→Met), 267 (Pro→Pro), and 290 (Gly→Gly) would enable accurate typing.

Methods for genotyping variants based on PCR-fragment analyses were also developed for *HLA-G* typing. Glycosylase-mediated polymorphism detection (GMPD) has been used to determine SNVs in codons 107 and 110 [93]. Briefly, exon 3 is amplified and used as the template for a secondary PCR using three normal dNTPs and a fourth modified dNTP (dUTP) that is variant-specific. The ^{32}P-labeled primer is designed so that the modified dNTP is the first incorporated base in the 3′ end. After amplification, DNA fragments are treated with uracil DNA-glycosylase, and the resulting apyrimidinic sites are cleaved, allowing detection of the variant through fragment length polymorphism using denaturing polyacrylamide gel electrophoresis and autoradiography [118]. Another technique is a multiplex platform based on a single base primer extension using fluorescently labeled dideoxynucleotide triphosphates (ddNTPs) for the identification of SNVs, which are detected by fragment size and fluorescence by capillary electrophoresis [71].

PCR-SBT based on Sanger sequencing has been widely used for *HLA-G* typing and characterizing its coding and noncoding regions. Most techniques are adapted from the original works of van der Ven and Ober [85], Yamashita and colleagues [86], and Hviid and colleagues [84]. In most studies, exons 2, 3, and 4 sequencing are usually performed with few interrogating intronic regions [81, 99, 100]. Other strategies include using long-range PCR fragments that overlap and span *HLA-G* full length, which allows for obtaining contiguous sequences and, thus, haplotype reconstruction [108]. Targeted approaches aiming at the nucleotide diversity of the noncoding regulatory regions 5′upstream regulatory region (URR) and 3′untranslated region (UTR) have also been developed for understanding evolutive aspects (i.e. balancing

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selection) maintaining higher nucleotide diversity in those regions compared with coding regions [100, 104–107]. Technical modifications from the original works of Ishitani and Geraghty [94], Fuji and colleagues [95], and Kirszenbaum and colleagues [96], were also instrumental in sequencing alternative mRNA transcripts yielding different HLA-G isoforms in pregnancy tissues [80, 97].

The use of NGS involves various methodological aspects, such as targeted regions, amplification strategies, library preparation, NGS platforms and technology, and bioinformatic tools. A significant drawback in parallel sequencing procedures is the accuracy of mapping reads, particularly when other HLA genes are processed together and mapped against the reference genome. Recognizing those challenges, Castelli and colleagues [110] proposed several bioinformatic tools for NGS applied to HLA-G: accurate read mapping (hla-mapper) [119], genotype calling (Genome analysis toolkit; GATK) [120–122], genotype quality check and imputation of missing alleles (vcfx) [123, 124]. Using this protocol, ~80% of heterozygous sites were phased by GATK, and the remaining, including imputed missed alleles, were inferred by the PHASE algorithm [125, 126]. The proposed protocol allowed comprehensive evaluation of the entire gene and extended haplotypes (5′URR/coding region/3′UTR). The protocol has been adapted with different library preparation and enrichment kits (HaloPlex/Agilent Technologies compared with Nextera XT/Illumina) and the inclusion of ancestry informative markers that determine the influence of population genetic structure in *HLA-G* diversity [111].

Commercial NGS set of reagents: NG-Mix (EFS, France), NGSgo-AmpX HLA-G (GenDx, The Netherlands), AlloSeq Tx17 (CareDx, USA), and proprietary software: PolyPheMe (Xegen, France), CLC Genomic Workbench (Qiagen, USA), Profiler (EFS, France), HLA or Omixon explore (Omixon Corporation, Hungary), and AlloSeq (CareDX, USA) have also been used for *HLA-G* sequencing and downstream analyses [112, 113, 115], including HLA typing and haplotype reconstruction from whole genome sequencing data [117].

The NGS platform based on Ion Torrent sequencing technology (library preparation: MNAse, New England Biolabs) was also used to obtain *HLA-G* haplotypes and diplotypes using the PHASE algorithm [113]. Another platform, based on single-molecule real-time sequencing technology (PacBio, USA), was used to obtain full-length consensus sequences of HLA-G [114, 127]. The advantage of this method over short-read sequencing is the ability to determine haplotype directly from the sequencing data. Of note, the technique performance was similar to previous approaches but allowed the identification of the novel G*01:01:01:01 allele that has a shorter poly T stretch (<25 bp) located downstream *HLA-G* and is associated with pre-eclampsia risk [114].

NGS offers significant advantages compared to PCR-SBT and PCR-SSOP in terms of resolution and cost-effectiveness. However, this higher resolution is accompanied by an exponential increase in DNA sequencing data produced by various platforms and technologies, which often remains unintegrated or unused for secondary analyses. In the context of *HLA-G*, a recent computation and bioinformatic developed by Castelli and colleagues [76] was applied in four datasets ($n = 4738$ individuals), resulting in the largest genetic diversity study of *HLA-G* and a resourceful list of NGS tools.

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25.5 *HLA-E* Typing Methods

The *HLA-E* gene is 4,686 bp long (NCBI reference sequence NM_005516.6) and is located between the *HLA-C* and *HLA-A loci*, two of the most polymorphic genes in the human genome. The gene and protein structure is akin to that of *HLA-A*, *-B,* and *-C* and consists of 8 exons and 7 introns. Specifically, exon 1 encodes the leader peptide, exons 2, 3, and 4 encode the α1, α2, and α3 extracellular domains, respectively. Exon 5 encodes the transmembrane region, while exons 6 and 7 encode the cytoplasmic tail (exon 7 contains 3 *Alu* sequences combined with a 5 bp deletion leading to translation termination). Exon 8 is not translated and is part of the 3′UTR (Figure 25.4).

HLA-E is relatively polymorphic compared with *HLA-F* and *HLA-G*. According to the IPD-IMGT/HLA Database (Report Release 3.57) [11], 366 alleles, 140 proteins, and 10 nulls have been reported. Despite high allelic and protein diversity found in HLA-E, earlier and more recent studies found two major alleles and proteins (E*01:01 and E:01:03) at nearly equal frequencies in multiple populations [128, 129]. The two alleles differ from each other by a single variant in codon 107 (AGG→GGG; Arg→Gly) located in exon 3 [130] (Table 25.6). Interestingly, linkage disequilibrium between *HLA-A* and *HLA-E* alleles (E*01:01 and E*01:03) has been reported [128]. However, recent surveys do not support previous associations, and the linkage disequilibrium between *HLA-E* and *HLA-F* alleles is unclear [70, 108]. Nevertheless, an association between *HLA-E* and *HLA-G* was found in isolated populations [71]. No linkage disequilibrium between *HLA-E* and class-I, -Ib, and II alleles has been found in admixed populations [72]. Despite the low number of variants necessary for genotyping and determining the most frequent *HLA-E* alleles, several techniques and protocols have been developed (Table 25.7). Earlier studies employed PCR-SSOP for typing HLA-E*01:01 and E*01:03 based on codon 107 [128], and later protocols improve the typing resolution by including codons 77 (AAC→AAT), and 82/83 (CGc/gGC→CGg/cGC) located in exon 2 [132].

PCR-RFLP protocols aim genotyping variants in codons 82/83, codon 107, and codon 157 (AGA→GGA; exon 3) using *BstUI*, *HpaII*, and *NlaIV* restriction endonucleases, respectively [90, 133]. Further, technical adaptations are made possible using enzyme isoschizomers. Noteworthy, variants in the codons 82/83 and 157 define the HLA-E*01:02 and E*01:04 alleles, which are considered rare or inexistent. ARMS-PCR was also developed for genotyping the variants in codons 77 (AAC→AAT; exon 2) and 107 [90].

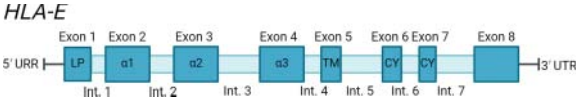

Figure 25.4 Schematic representation of *HLA-E* gene organization and structure. The gene has eight exons separated by seven introns. Exons 6 and 7 encode the cytoplasmic tail (exon 7 has three Alu sequences combined with a 5-base pair deletion leading to translation termination). Exon 8 is not translated and is part of the 3′UTR. 5′URR, upstream regulatory region. 3′UTR, untranslated region; LP, leader peptide; TM, transmembrane domain; CY, cytoplasmic tail; Int, intron.

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Table 25.6 Nonconservative and conservative amino acid changes in exons 2–4 used for typing common *HLA-E* alleles.

Allele	Exon 2	Exon 3	Exon 4	Freq.[a)]	Freq.[b)]
	77	107	189		
E*01:01:01 (Ref)	AAC	AGG	GTG	0.548	0.572
E*01:03:01		GGG		0.152	0.112
E*01:03:02	AAT	GGG		0.296	0.281
E*01:03:05		GGG	GTC		0.023

HLA-E alleles showing frequency above 1%.

a) Estimated *HLA-E* allele frequency over 2.5 million samples (Sauter et al. 2021 [129]).

b) Estimated *HLA-E* allele frequency in unrelated Brazilians ($n = 420$) (Ramalho et al. 2017 [131]). Ref, reference allele. Nonconservative and conservative amino acid changes according to the IPD-IMGT/HLA Database (Release 3.57 (2024–2107) Version Report – Build 6fc03c9; Barker et al. 2023 [11]).

PCR-SSP protocols are designed to genotype variants in codons 66, 77, 83, 107, 131, 157, and 292. Despite including more variants compared to other techniques, it has been shown that codons 77 and 107 are the most informative [135] and allow the identification of the most frequent *HLA-E* alleles (Table 25.6). In this context, a Taqman assay (Assay ID: C_175677552_10, Thermo Fisher Scientific, USA) for genotyping the variant in codon 107 was developed [136].

Because the *HLA-E* coding regions are not polymorphic, PCR-SBT has been employed to characterize the diversity within regulatory and intronic regions. *HLA-E* full length was investigated by PCR-SBT using long-range PCR fragments to obtain contiguous sequences [108, 138] or sequencing overlapping fragments [139]. Noteworthy, the sequencing of exons 1, 2, 3, and 4 confirmed the lack of variability in the coding region and revealed equal frequencies of the E*01:01 and E*01:03 alleles in both healthy and pathological contexts [72, 101, 137, 140]. Also, balancing selection at exon 3 (codon 107) is likely shaping HLA-E dimorphism, while exon 4 and the noncoding regions are under purifying pressure [139]. Also, the 3′UTR showed lower variation than the coding region, suggesting that this region has crucial feature for tissue-specific regulation via post-transcriptional mechanisms [141].

NGS use in *HLA-E* research revealed some previously unrecognized diversity compared to other methods. Different strategies have been developed including sequencing of different *HLA-E* genomic regions and fragment length (2.8–7.6 kb), parallel sequencing of other *loci*, variations in NGS platforms (short and log-reads), and sample size. Castelli and colleagues proposed a strategy to evaluate full-length *HLA-E* variability by NGS, which was later adapted for sequencing single or multiple MHC-Ib genes (*HLA-G* and *-F*) [110, 142]. Subsequent NGS data confirmed that the two HLA-E proteins (E*01:01 and E*01:03) account for most *HLA-E* encoded molecules in most populations analyzed. Also, variability in the promoter region is higher than the coding and 3′UTR, with the latter more variable than the coding

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Table 25.7 DNA-based methods developed for *HLA-E* typing.

Method	Target regions for *HLA-G* typing	References
PCR-SSOP		
	HLA-E cDNA: Exon 2 and 3	Geraghty et al. 1992 [128]
	Exons 2 and 3	Gómez-Casado et al. 1997 [132]
PCR-RFLP		
	Exons 2 and 3	Matte et al. 2000 [90]
	Exon 3	Tamouza et al. 2005 [133]
ARMS-PCR		
	Exons 2 and 3	Matte et al. 2000 [90]
PCR-SSP		
	Exons 2 and 3	Grimsley et al. 2002 [134]
	Exons 1, 2, 3, and 5	Antoun et al. 2009 [135]
	Exon 3	Tamouza et al. 2005 [133]
		Lauterbach et al. 2012 [4]
RT-PCR		
	Exon 3	Paquay et al. 2009 [136]
PCR-SBT		
	Exons 2 and 3	Gómez-Casado et al. 1999 [81]
		Lauterbach et al. 2012 [4]
		Carvalho dos Santos et al. 2013 [137]
		Castro et al. 2019 [72]
	Full gene	Pyo et al. 2006 [108]
		Olieslagers et al. 2017 [138]
	Exons 2, 3, and 4	Paquay et al. 2009 [136]
	Exons 1, 2, and 3	Antoun et al. 2009 [135]
	Exons 1, 2, 3, and 4	Veiga-Castelli et al. 2012 [139]
	Exon 3	Ferguson et al. 2011 [101]
	Exon 1 to 3′UTR	Veiga-Castelli et al. 2013 [140]
		Felício et al. 2014 [141]
NGS[a)]	Full gene (5 kb)	Castelli et al. 2015 [123]
	Exons 2 to exon 7 (2.8 kb)	Carlini et al. 2016 [70]
	Upstream/downstream HLA-E (7.6 kb)	Ramalho et al. 2017 [131]
		Sonon et al. 2018 [142]
	Exons 2 and 3	Sauter et al. 2021 [129]
	Full gene (3.3 kb)	Paech et al. 2021 [143]
	Whole genome sequencing	Williams et al. 2021 [117]
		Ormandjieva et al. 2023 [52]

PCR, polymerase chain reaction; PCR-SSOP, sequence-specific oligonucleotides probes; PCR-RFLP, restriction fragment length polymorphism; ARMS-PCR, Amplification-refractory mutation system PCR; PCR-SSP, sequence-specific primer; RT-PCR, real-time PCR; PCR-SBT, sequencing-based typing; NGS, next-generation sequencing; cDNA, complementary DNA; 5URR, upstream regulatory region; 3UTR, untranslated region.

a) NGS platforms, Castelli et al. 2015, Carlini et al. 2016, Ramalho et al. 2017, Sonon et al. 2018: Illumina MiSeq. Sauter et al. 2021: Illumina MiSeq, HiSeq, and NovaSeq. Paech et al. 2021: Illumina and PacBio single-molecule real-time sequencing. Williams et al. 2021: Illumina. Ormandjieva et al. 2023: Illumina MiSeq.

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[123, 142]. NGS has also been used for parallel sequencing of *HLA-E* (exons 2 and 3) and highly polymorphic *MIC* genes in the largest cohort reported, comprehending over 2.5 million individuals [129]. The workflow and analysis of a large NGS dataset was possible by using the typing software nexType [144]. In this large sample, 345 new *HLA-E* alleles were found, of which 234 alleles were fully re-sequenced (5′URR to 3′UTR) by using short-read Illumina and long-read PacBio sequencing to obtain fully phased sequences. Noteworthy, for most samples, both methods yielded consistent contiguous sequences. Regarding inconsistencies between NGS technologies, the dual redundant reference sequencing (DR2S) pipeline, which handles both Illumina and PacBio data, was used to generate accurate consensus sequences [143, 145]. Such approaches demonstrated the advantage of using multiple NGS platforms over traditional Sanger methods when phasing and haplotype reconstruction are clinically relevant.

25.6 *HLA-F* Typing Methods

The *HLA-F* was first cloned by Geraghty and colleagues [146]. HLA-F is a β2-microglobulin-associated protein of 41 kDa. The smaller size compared to typical HLA-I proteins corresponds to a shorter cytoplasmic domain due to the exclusion of exon 7 from the mature mRNA. *HLA-F* gene is located ~88 kb telomeric to *HLA-G* and has a 3,863 bp length (NCBI Reference sequence NM_001098479.2). The gene organization is similar to other HLA-I genes (Figure 25.5). Exon 1 encodes the leader peptide, and exons 2, 3, and 4 encode the extracellular domains α1, α2, and α3, respectively. While exon 5 encodes the transmembrane domain, and exon 6 is the cytoplasmic domain. Exon 7 has an altered splice acceptor site that removes it from the mature mRNA [147].

The precise function of *HLA-F* still needs to be better understood. Recent studies have found that HLA-F can selectively bind and present peptides [148, 149], but peptides may not be necessary for stabilizing HLA-F. Its membrane expression seems independent of both TAP and tapasin [150]. Further, HLA-F molecules without peptide and lacking β2-microglobulin function as open conformers that are recognized by immune cells [151, 152].

HLA-F diversity is represented by four alleles, F*01:01, F*01:02, F*01:03, and F*01:04, F*01:01 representing more than 80% of allelic diversity. According to the IPD-IMGT/HLA Database (release 3.57) [11], 94 alleles, 19 proteins, and 2 nulls were reported. Methods for typing HLA-F have focused on determining a few

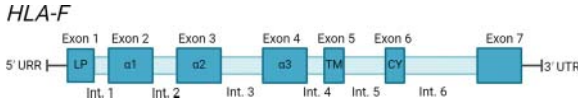

Figure 25.5 Schematic representation of *HLA-F* gene organization and structure. The gene has seven exons separated by six introns. Exon 7 has an altered splice acceptor site that removes it from the mature mRNA. 5′URR, upstream regulatory region; 3′UTR, untranslated region; LP, leader peptide; TM, transmembrane domain; CY, cytoplasmic tail; Int, intron.

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Table 25.8 Nonconservative and conservative amino acid changes in exons 1, 2, and 4 used for typing common *HLA-F* alleles.

Allele	Exon 1		Exon 2		Exon 4
	−9	−1	39	50	251
F*01:01:01 (Ref)	GCC	GCG	GAC	CCG	TCT
F*01:01:02		GCA			
F*01:02:01	GTC				
F*01:03:01					CCT
F*01:03:02			GAT		CCT
F*01:04:01				CAG	

Nonconservative and conservative amino acid changes according to the IPD-IMGT/HLA Database (Release 3.57 (2024–2107) Version Report – Build 6fc03c9; [11]).

variants located in exons 1, 2, and 4 (Table 25.8**)**. Despite the low frequency of *HLA-F* null alleles; the F*01:14N allele has been found in a Japanese population at a frequency of 0.56%. The null allele is caused by a premature stop codon in exon 3 located in codon 118 (TAC→TAA) [153]. This allele was first detected by contrasting DNA banding patterns using PCR-SSCP and further identified by PCR-RFLP using the restriction endonuclease enzyme *RsaI* (Table 25.9). Other techniques based on single-allele specific primes, such as PCR-SSP and PCR-LDR (ligase detection reaction) were developed for typing *HLA-F* alleles (F*01:01, F*01:02, F*01:03, and F*01:04) [154, 155]. PCR-LDR uses a denatured DNA fragment of *HLA-F* previously amplified by PCR. Then, allele-specific primers of different lengths with the discriminating base at the 3′end and a common fluorescent labeled primer complementary to the upstream sequence of the SNV are hybridized with the DNA in the presence of a thermostable DNA ligase. The DNA ligase ligates the discriminating primer and labeled probe in a single DNA fragment. After hybridization cycling reactions, the genotype is determined based on fragment length by capillary or gel electrophoresis.

PCR-SBT was also developed for typing and characterizing *HLA-F* variability. By sequencing *HLA-F* cDNA, the F*01:01:02 allele was defined by a synonymous mutation in exon 1 residue −1 (GCG→GCA), and it was further confirmed that *HLA-F* transcripts lack the exon 7 [156]. Similar methodological strategies also identified and reported informative variants for accurate *HLA-F* typing [157]. PCR-SBT was also applied to investigate *HLA-F* at higher resolution in several cell lines and to provide pieces of evidence of linkage disequilibrium between *HLA-F* and other nonclassical HLA-I genes [108]. Lastly, sequencing strategies targeting exons 1–3 have been used for determining *HLA-F* allele frequency and linkage disequilibrium patterns with other MHC loci in bone marrow donor registries [72, 158].

NGS strategies significantly improved our understanding of *HLA-F* genetics [70, 124]. A NGS workflow for *HLA-F* has been developed by Lima and colleagues [124]. Commercial reagent kits and software have also been developed for parallel

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Table 25.9 DNA-based methods developed for *HLA-F* typing.

Method	Target regions for *HLA-F* typing	References
PCR-SSCP		
	Exons 2 and 3	Uchigiri et al. 1997 [153]
PCR-RFLP		
	Exon 3	Uchigiri et al. 1997 [153]
PCR-SSP		
	Exons 1, 2, and 4	Pan et al. 2013 [154]
PCR-LDR		
	Exons 1, 2, and 4	Zhang et al. 2012 [155]
PCR-SBT		
	HLA-F cDNA	He et al. 2004 [156]
	HLA-F cDNA Exon 2	Moscoso et al. 2006 [157]
	Full gene	Pyo et al. 2006 [108]
		Pan et al. 2013 [154]
	5′URR to exon 3	Manvailer et al. 2014 [158]
	Exons 1, 2, and 3	Castro et al. 2019 [72]
NGS[a)]		
	Full gene	Carlini et al. 2016 [70]
		Ormandjieva et al. 2023 [52]
	Upstream/downstream *HLA-F*	Lima et al. 2016 [124]
		Sonon et al. 2018 [142]
		Buttura et al. 2019 [159]
	Whole-genome sequencing	Williams et al. 2021 [117]

PCR, polymerase chain reaction; PCR-SSCP, single-stranded conformation polymorphism; PCR-RFLP, restriction fragment length polymorphism; PCR-SSP, sequence-specific primer; PCR-LDR, ligase detection reaction; PCR-SBT, sequencing-based typing; NGS, next-generation sequencing; cDNA, complementary DNA; 5URR, upstream regulatory region; 3UTR, untranslated region.

a) NGS platforms: Carlini et al. 2016, Ormandjieva et al. 2023. Lima et al. 2016, Sonon et al. 2018, Buttura et al. 2019: Illumina MiSeq. Williams et al. 2021: Illumina.

sequencing and typing *HLA-F* and other MHC genes [52]. Lastly, proprietary software was also developed for typing *HLA-F* and other MHC genes from whole genome sequencing data with minimum ambiguity [117]. With increasing sequencing coverage and typing resolution novel alleles were found in relatively high frequencies in human populations. For example, the *HLA-F**01:01 allele is mainly represented by F*01:01:01 and F*01:01:02, and together represent over 80% of *HLA-F* alleles found in human populations [70, 124, 142]. Of note, a single

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synonymous mutation in exon 1 discriminates HLA-F*01:01:01 between F*01:01:02 [156], and a potential association between these two allele with squamous cell carcinoma risk was reported [52]. Later studies exploring NGS datasets and RNAseq found that the F*01:01:02 allele is usually associated with a specific distal promoter and a 3′UTR haplotype, and is associated with higher mRNA expression [159].

25.7 Technical Aspects for the Detection of Anti-MICA Antibodies in Transplantation Patients

25.7.1 Introduction

Acute and chronic rejections remain the primary cause of organ allograft loss. Humoral mechanisms were considered mainly responsible for acute rejections when anti donor HLA antibodies were present before grafting. However, increasing evidence suggests that a humoral response to donor alloantigens other than classical HLA could play an important role in organ rejection [160–163]. The majority of these antibodies associated with organ transplantation appeared to be correlated with a harmful role on graft survival, although protective antibodies have also been described [164, 165]. However, non-HLA antibodies do not affect transplant outcome only in humoral anti-allograft responses but also by modulation of the immune system. Zhou et al. [160] proposed that "in addition to the adaptive immune response of T and B cells against an alloantigen, MICA is also capable of setting in motion the mechanisms of innate immunity. Co-stimulation by engagement of NK cells might have the effect of potentiating the T and B cell response". It was hypothesized that MICA antigens play a role in human allograft rejection by activating both humoral as well as cellular mechanisms [166]. Data from the 14th International Histocompatibility and Immunogenetics Workshop confirmed this hypothesis and indicate, that MICA antibodies were significantly and independently associated with reduced kidney allograft survival in deceased donor grafts, providing strong evidence for the involvement of these antibodies in graft rejection [167].

25.7.2 Determination of Anti-MICA Antibodies

Current methods using purified and recombinant MICA molecules have improved the sensitivity, accuracy, and reproducibility of anti-MICA antibody detection in the sera of transplanted patients. At the beginning, anti-MICA alloantibodies were detected using an ELISA assays with insoluble recombinant MICA alleles produced in *Escherichia coli* [168]. Based on the ELISA experiences, recent studies have used assays to detect alloantibodies against MICA alleles by Luminex™ technology with soluble MICA alleles produced in insect cells [160, 162, 166–169].

The Luminex assays enable the detection of antibodies against MICA alleles bound to fluorescently labelled polystyrene microbeads. The basic principle of the Luminex technology is the ability to measure multiple analytes simultaneously in

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a single reaction well. Molecular reactions take place on the surface of polystyrene microbeads (microspheres) that are color-coded using a blend of different fluorescent intensities of two dyes. When using this method, over 100 distinct microbead sets can be created. The microbeads act as molecular carriers that capture samples and are then tagged with a fluorescence-labeled reporter binding to the captured sample on the microbead. Afterwards, the microbeads are transported into the Luminex analyzer by using a precision sheath fluid stream to align them in single file, where they pass through two lasers, similarly to classical flow cytometry. One laser excites the colors inside the microbeads to identify which microbead is currently being read. The second laser excites the color on the microbead surface, i.e. the labeled reporter tag. Finally, the color signals are detected by an advanced optical system, and the signals are processed into data for each reaction [170]. Today the Luminex-based antibody screening technology is widespread used in laboratories affiliated to organ transplantation programs and enables both screening (i.e. the definition of positive or negative antibody status) and antibody identification with high sensitivity and specificity. The commercially available test kits use purified antigens immobilized directly on the microbeads. Anti-MICA antibodies of the IgG isotypes, which are bound to the microbeads, are detected by a secondary IgG-specific antibody, which is conjugated with R-phycoerythrin (PE). The mean fluorescence intensity (MFI) is the relevant read out. Today, samples can be tested for antibodies against MICA using commercially available test kits from two vendors (LABScreen™-technology, One Lambda/Thermo Fisher, Canoga Park, CA, USA and LSA-MIC™ Assay, Lifecodes/Immucor/Werfen, Waukesha, WI, USA). Samples will be measured on Luminex 200 or FM3D machines and subsequently analyzed using company specific software.

The LABScreen-Mixed Beads (One Lambda/Thermo Fisher) assay comprises 17 beads specific for HLA antibodies and two for MICA, respectively. Analysis of the raw data based on mean MFI is done using the HLA-Fusion™ Software (One Lambda/Thermo Fisher, Canoga Park, CA, USA). Reactions against each bead are scored as 1 (negative), 4 (undetermined, i.e. a grey zone not defined as true positive) or 8 (positive) based on positive/negative ratios of control values of each test batch. The thresholds for the definition of "undetermined" and "positive" are given in the manufacturer's instructions. However, after careful evaluation and validation individual cutoffs can be established. For studies performed in our laboratory a normalized background ratio above 3.0 was defined as positive and a range between 2.0 and 3.0 as undetermined. Finally, a ratio below 2.0 was defined as negative [171, 172].

The LABScreen-Single Antigen Beads assay for MICA by One Lambda comprises ten beads specific for the ten most frequent MICA alleles (MICA*001, *002, *004, *008, *009, *012, *017, *018, *019, and *045). The LSA-MIC assay by Immucor is also a Single Antigen Beads assay and comprises 28 beads specific for 28 different MICA Alleles (MICA*001 to *051). Immucor recommends using the MATCH IT™ software for analysis. For both assays, it is generally recommended to treat sera with EDTA prior to the Luminex SAB testing [173]. The positive cut off value for MFI in the Single Antigen Bead assay is set as 1,000 MFI and the positive MFI cut off value for is also set as 1,000 MFI for both companies.

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Part IV

Regulatory Aspects

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26

Regulations in Histocompatibility and Immunogenetics: Accreditation, External Proficiency Testing, Quality Management

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26.1 Introduction

Solid organ (SO) and hematopoietic stem cell (HSC) transplantation is among the most dynamic fields of medicine, whose success relies strongly on skilled Histocompatibility and Immunogenetics (H&I) laboratories. Figure 26.1 depicts some of the milestones in transplantation (in red) and in H&I (in green) over the past 70 years, highlighting how the clinical and laboratory aspects are closely interlaced [1–4].

Transplantation programs are multidisciplinary and usually comprise many hospitals at national and international levels, entailing that donor samples, organs, HSCs, HLA typing, HLA antibody, and clinical data are shared among different transplant centers, H&I laboratories, and registries. For these exchanges to take place harmoniously, a significant degree of standardization must be achieved [5].

To support clinical and laboratory features of transplantation programs and with the aim of encouraging standardization and the highest possible safety and quality levels, over the years, laws, regulations, guidelines, and standards have been published by national governments, the European Union, the Council of Europe, and scientific societies (see the lower part of Figure 26.1).

In this chapter, we will illustrate the role that Quality Management Systems (QMS) and accreditation programs have had on the development of H&I laboratories.

26.2 Legal Requirements

Laboratories and the clinical services they support must comply with all relevant laws and regulations. These requirements are established by national governments although in some cases the same or similar laws may be in place in different jurisdictions, e.g. through the transposition of European Union Directives into national laws.

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Figure 26.1 Clinical (red), H&I (green), and regulatory (blue) milestones in transplantation.

While generally there are no specific laws relating to laboratory testing, the field of transplantation is regulated, in particular with laws which govern the donation and transplantation of cells or organs. Laboratory services must ensure that the services they provide are compliant with any legal requirements placed on the clinical practices they support. Additionally, compliance with laws which govern health and safety, employment, financial practices, etc., must also be complied with.

26.3 National Guidelines

Guidelines are an instrument to set general requirements for the proper running of a laboratory or clinical service. They are frequently produced by a national medical authority or recognized professional body. Guidelines usually describe a minimum standard and do not have the same restrictions as a legal requirement. Guidelines are important national tools and can be updated much faster than laws. Due to this, laboratory working can respond more effectively to changes in technology or clinical knowledge.

26.4 Standards

Usually, most standards are not mandated by law and are meant to act on a voluntary basis. However, some standards which achieve widespread use or acceptance may be adopted by regulators as a legal requirement in particular domains. In the latter case, they become mandatory for laboratories.

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Standards can make cooperation between different organizations much easier. In that situation, compliance with the same set of standards can help to ensure a common understanding of the requirements which all parties need to meet to provide safe clinical practices.

In general, there are two main types of standards which are applied to laboratory and clinical practices:

a) international formal standards;
b) professional body affiliated standards.

Both types of standards result from expert groups who are working in the nominated discipline.

26.4.1 International Organisation for Standardization

The applicable ISO standards in medical laboratories are *ISO 15189:2022 Medical Laboratories – Requirements for Quality and Competence*. ISO standards represent a rigorous set of standards which are recognized internationally. The standards focus on the structure and performance of a QMS, including quality assurance, risk analysis, PDCA-development, quality of services, and evidence-based practices [6]. The standards **are generic** and applicable to many different medical laboratory specialties.

From certain points of view in H&I, ISO standards can have some disadvantages:

1) ISO standards are written for all medical laboratories, which means less specificity for H&I.
2) Since ISO is a large and worldwide operating Non-Governmental Organisation (NGO), changes to standards are a laborious act and can take several years before being implemented.
3) Accreditation to ISO standards alone is so far not fully accepted in some H&I-related organizations, e.g. Eurotransplant, donor matching or procurement organizations, and other medical institutions like FACT and JACIE [7, 8].

26.4.2 Professional Body Standards

Standards produced by professional bodies **are discipline specific** with a strong focus on related medical topics, and generally take into consideration the scientific/clinical evidence base to support the requirements they define.

This has several advantages:

1) Specific demands can be much more detailed than in other regulations and are more discipline specific than in laws or ISO standards.
2) Changes that result from modern research or medical experience can be incorporated much faster into new/updated standards compared to bigger organizations.
3) Standards can be recognized by other scientific/medical bodies in a national or international context supporting activities which cross national boundaries.

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While the focus of these standards is on discipline-specific requirements, many professional body standards also include some or many of the elements required for a QMS.

26.5 Elements of Quality Management Systems

The term "*Quality*" in the healthcare context has been defined by the Institute of Medicine (now the National Academy of Medicine) in the following manner: "the extent to which health services for individuals and populations increase the probability of desired health outcomes and conform with current professional knowledge" [9]. Another more concise definition of Quality in healthcare is "doing the right things, for the right patient, at the right time, in the right way to achieve the best possible results". In more recent years, there seems to be a consensus that quality involves safety, effectiveness, appropriateness, responsiveness or patient-centered care, equity or access, and efficiency [10].

As we will see in the following sections, there are different ways an H&I laboratory can approach "*Quality*", but most, if not all, models apply elements of QMS, by which we mean *"the set of policies and procedures for establishing and implementing the intentions of the organisation with respect to quality"* [11].

For an overview of central QMS elements, please refer to Appendix 26.A.

For a QMS to be effective, senior management must be convinced of its utility, must allocate the necessary resources, and should encourage practices related to quality. This will allow staff to develop an "awareness" of quality which is at the basis of a successful QMS.

Appendix 26.B lists some of the elements of QMS, with their definition and their application in three different quality models: the European Federation for Immunogenetics (EFI) accreditation program, the ISO 15189:2022 scheme, and the ISO 9001:2015 standard.

26.6 Practical Considerations for a QMS

26.6.1 Gap Analysis

When a laboratory is starting to establish a QMS or trying to improve a given QMS, it is of importance to get an overview of which regulations are relevant to the laboratory working system. This includes a gap analysis, analyzing what is already in place and what more is required.

A combined model of requirements is given in Figure 26.2 and addresses the different needs for the QMS when covering all aspects. From this, one can also deduce that it might include both ISO standards and medical specific standards depending on the context.

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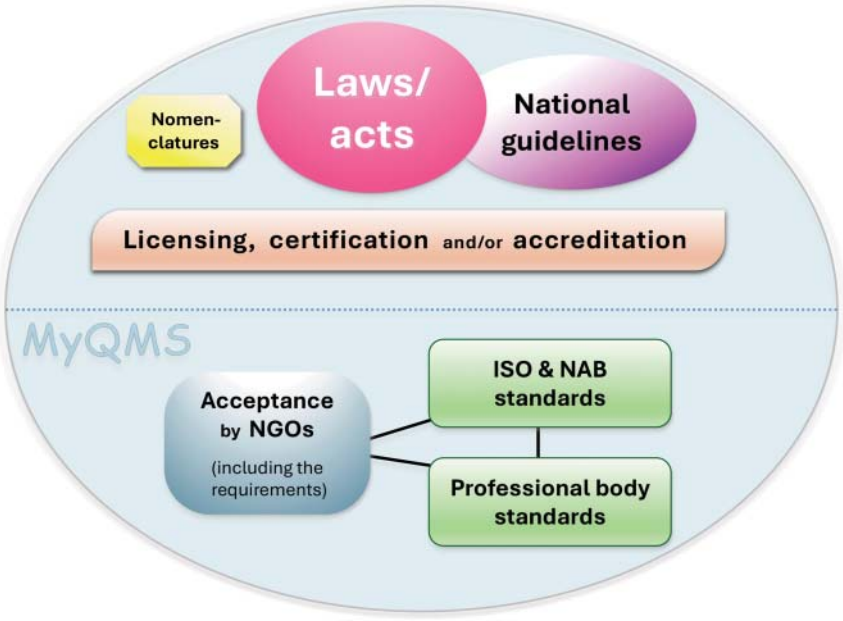

Figure 26.2 Gap analysis of local regulations for your laboratory. When taking everything into account, these requirements will assign the outline of your QMS.

26.6.2 Basic Structures for an Institutional QMS

Quality does not evolve by itself or by collecting fact sheets and reports. Everything must be planned, tested, corrected, monitored, and interpreted in context. All aspects must be documented to ensure that all individuals involved in the provision of the service have access to controlled policies and procedures and work according to the requirements of the QMS. Last but not least, there should be a continuous cycle of improvement.

Over time, many models were tested in laboratories and manufacturing companies. From these experiments, one principle was chosen again and again and has become accepted as a common method for maintaining and improving QMS: the Deming cycle.

Between 1940 and 1985, *William Edward Deming* developed the idea of continuous improvement in quality development. He divided the cycle into four major parts and added safeguard steps to the laboratory processes [12]. The names of the parts are "plan", "do", "check", and "act". The cycle is thereafter also known as the PDCA cycle. In 2008, the PDCA cycle also became a must in ISO Standard 9001 [13].

Deming's ideas start from a basic principle: Standstill means regression. If there is no development in your processes, time will erode your QMS, and if you fail to improve, you have already lost the race.

This idea is often symbolized by a wheel on a ramp (Figure 26.3). The QMS needs steady improvement. If you don't use your energy to push the wheel upward, it

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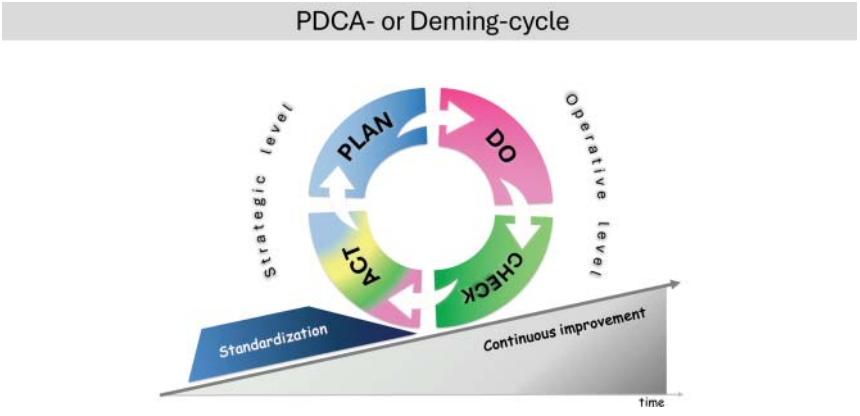

Figure 26.3 Deming- or PDCA-Cycle Model [12, 13].

surely runs down the ramp. Therefore, standardization is an aspect of securing advancements after each cycle.

26.6.3 PDCA: Cycle Descriptions

In the following overview, the four parts of the process are given in more detail. Relevant questions are listed to give a better overview to what has to be answered in this part and summarized in a highlighted box with the typical question for the given chapter.

26.6.3.1 P – Plan

1) Within this part, you specify the aims:
   What are the goals of the institute?
   What do we want to reach?
   - diagnostics and/or blood cell production
   - the general institutional policies

   What are the resources?
   - staffing
   - laboratory rooms and equipment
   - funding, e.g. for reagents, maintenance, and backups
   - IT and/or bioinformatic requirements

2) From the gap analysis, you will realize which requirements are necessary to become recognized by others:
   - Which requirements are mandated by laws and national guidelines?
   - Do we need certification +/− accreditation +/− licensing?
   - Decision: Do we have to work according to ISO-standards, professional body affiliated standards, or both?

All items are relevant for your work and give important information on how to establish a QMS.

The summarizing question to be asked here is: **Do we do the RIGHT THINGS?**

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26.6.3.2 D – Do

Starting from your basic QMS, the next step is to define two parts:

a) QM-part:
   - provide a format/structure for your documents and for document control
   - decision either to use a paper-based system or an electronic QM software
b) Laboratory part: Need of descriptions for diagnostic and production processes:
   - process instructions:
     - defining general procedures (e.g. how are pipettes calibrated)
     - defining the number and sort of external proficiency testing schemes (EPTs)
     - how long are documents, checklists, etc., to be archived?
   - operating instructions:
     - describing a given method in detail (which instrument, reagent, protocol, and quality control, where, and how to archive these)

The typical question to be asked here is: **Do we do our things RIGHT?**

26.6.3.3 C – Check

This part of the cycle defines methods and procedures on how to check the documented processes from the Plan and Do cycle. It checks whether the descriptions in the SOPs have been done correctly and with the expected controls.

Quality control and steering aspects:

- Do the written procedures fit the expected format and are they complete?
- Were aspects of validation, verification, risk assessment, maintenance, and calibration performed as requested by the national guideline/standard?
- Did the laboratory complete the expected EPTs with success?
- Were "mistakes" listed and deviations corrected? Were there preventive actions to avoid a recurrence of the same mistake?
- Were quality controls done completely and documented as expected?
- Has training and education been done as predicted in the yearly overview?
- Do we have customer complaints? Is there a statistical examination?

The typical question to be asked here is: **Have we done our right things RIGHT?**

26.6.3.4 A – Act

The fourth part of the cycle is meant for the overall management, together with all responsible and leading coworkers from all areas. In repeated meetings, they should discuss about results that were found in a defined time period, outcomes, problems, and improvement.

Finally, two important answers should result from this meeting:

a) "Should we safeguard steps in our processes in order to keep the standard?" (HOLD)
b) Should we adopt new practices to become safer, more effective or any better in certain processes? (IMPROVE)

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c) Should we stop any process due to unacceptable results or insufficient resources? (STOP)

The main idea is to find objective arguments if processes have "weak or unsure" elements, are inadequate, or run ineffectively.

The typical question to be asked here is:

Can we make our things any BETTER and **do we still do the RIGHT things?**

26.7 Licensing, Accreditation, and Certification

When preparing for your quality manual, it is important to know which standards the laboratory is working with. In this situation, it might be possible that more than one set of standards is in use.

In the global setting, partners must trust each other, and they need to know that all parties are working with reliable methods and processes. Unlike laws, acts, or national guidelines, which must be complied with, accreditation and certification are voluntary activities. However, in many cases, it is a precondition of the healthcare systems and/or funders for laboratories to be accredited.

So, without these qualifications, a laboratory may not be able or allowed to do specific diagnostic work for a defined community, or their data might not be accepted for national and international matching or procurement services.

a) **Licensing** represents governmental control for specific tasks, e.g. production of blood cell products. In some countries, blood products have the status of a pharmaceutical prescription product, in others as a controlled medicine. Only institutions that can show conformity with the high GMP-/or GLP-standards can get such a manufacturing license. Therefore, they are under permanent regulation and must accept external onsite peer-reviewing. Very often, licensing illustrates the strongest type of external evaluation.
b) **Accreditation** represents another type of external control, which is usually done by a single officially recognized organization, in many countries called National Accreditation Body (NAB). In contrast to licensing, accreditation is voluntary.

   When NABs are members of the International Laboratory Accreditation Cooperation (ILAC) and the International Accreditation Forum (IAF), they are recognized worldwide, including national governments. In some countries, accreditation to recognized standards can be an additional requirement mandated by government as a precondition for certain categories of laboratory work.

   In some jurisdictions, the term accreditation has a protected status and can only be used by NABs, e.g. in the European Union, whereas in other regions, the term accreditation may be used by other organizations in addition to NABs. For the purposes of this chapter, we will use the term certification in relation to schemes for assessment and approval against professional body standards by other organizations (e.g. EFI [14, 15], ASHI [16], FACT-JACIE [7]), although the title of the scheme may include the term accreditation.

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c) **Certification** is also a voluntary process. They are usually controlled by so-called Certification Bodies (CBs) or 3rd party institutions, including professional bodies. Certification by professional body affiliated assessment against discipline specific standards is of particular relevance in specialized medical fields and in H&I is an aid to supporting global collaboration and cooperation.

For a synopsis of these three programs, please refer to Appendix 26.D.

26.8 EFI Accreditation Program

The EFI accreditation program was established in 1995 with the aim of providing assurance that the H&I laboratories meet the requirements of the EFI Standards. Currently (in 2024), there are over 270 accredited laboratories mostly in Europe but also in South America, Africa, and Asia, and details on the program can be found on the EFI website. Since EFI Standards have historical roots to ASHI Standards, both accreditation committees work with the same basic ideas and concepts [14-16].

As shown in Appendix 26.B, the EFI standards address the majority of the QMS elements, with the exception of risk-based thinking, internal audits, and the concept of continuous quality improvement. A section of the EFI standards (section C) is dedicated to quality assurance, a term which can be defined as "all activities performed in the laboratory aimed at the maintenance of a system to ensure that the overall performance is of required quality, and to detect shifts or trends in the quality of products that may require attention" [11]. Section C covers topics such as management, facilities, equipment, reagents, and preanalytical processes.

EFI standards are characterized by being very specific on the technical aspects of methods (CDC, antibody testing and crossmatching, ELISA, flow cytometry, nucleic acid analysis, bead array, SSP, SSOP, Sanger sequencing, NGS, hematopoietic chimerism engraftment, and other immunological markers), clinical applications (renal/pancreas transplantation, other organ transplantation, HSC transplantation, transfusion, and disease association studies), and post analysis processes (e.g. terminology, records, and reports).

One topic which is very detailed in the standards is External Proficiency Testing (section D), which plays a pivotal role in quality assurance. Recently, *C. Voorter and coauthors* published a paper on 19 years of international external proficiency testing for high resolution HLA typing, showing a significant improvement in the performance of the participating laboratories over the years; since 2017, more than 98% of EPT samples are typed correctly [17].

In the fastly evolving field of H&I, the EFI accreditation program can be seen as a framework for clinically significant advancements to be processed and brought to the routine transplantation immunology laboratories [5].

A similar program to the one briefly described is that of the *American Society for Histocompatibility and Immunogenetics (ASHI)* which has its own standards and accreditation process, and whose aim is "to evaluate laboratory personnel, procedures and facilities to determine, if they are in compliance with those published Standards of ASHI and with those Standards of organizations by which ASHI

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is deemed and which apply to the activities of that laboratory. Laboratories will be evaluated for the technologies utilized and, if applicable, the clinical services provided" [16].

26.9 ISO Standards

26.9.1 ISO 15189:2022 – Medical Laboratory Requirements

The aim of this ISO norm is to promote the welfare of patients and satisfaction of laboratory users through confidence in the quality and competence of medical laboratories. An overview on international cooperation and local governmental appointment is given in Appendix 26.C.

Laboratories that wish to follow this scheme must plan and implement actions to address risks and opportunities for improvement, with the following key endpoints:

1) increasing the effectiveness of the management system,
2) decreasing the probability of invalid results, and
3) reducing potential harm to patients, laboratory personnel, the public, and the environment.

Practically, all elements of QMS (Appendix 26.B) are covered by ISO 15189:2022. One of the main differences between the EFI standards and ISO 15189 is that while the former addresses specifically H&I laboratories, the latter is intended for use throughout the currently recognized medical laboratory disciplines.

It should be highlighted that some governmental or professional body institutions, e.g. in the United Kingdom, Germany, and France, consider – at least in parts – ISO 15189 and EFI accreditation to be complementary, and therefore require that their H&I laboratories are accredited with both systems, to cover the different QMS elements.

For a first insight and preparation of a QMS, the World Health Organisation (WHO) is providing a pdf handbook, additional worksheets, and protocols for a "Laboratory Quality Management System" according to ISO 15189, which is still a good compendium to start an own QMS system [18].

26.9.2 ISO 9001:2015

ISO 9001 is part of the ISO 9000 family of quality management standards and specifies requirements for a QMS in any type of organization providing products or services. Because of its wide spectrum of application, this standard is necessarily generic [13]. Since this ISO Standard is very widespread, e.g. a basic part of the ISO 15189 Standards, it is also called *the mother of QMS standards*.

Appendix 26.B shows that most QMS elements are addressed by this norm, but as expected, it does not contain technical standards on clinical laboratories, as can be found in the other two models considered in this chapter. [13] In some countries (e.g. Italy), in which the implementation of ISO 15189 is not yet compulsory, it is quite common for EFI accredited H&I labs to be both EFI and ISO 9001:2015 accredited.

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In the past few years, there has been an increase in literature related to different aspects of the implementation of quality systems in medical laboratories. *Chaudhry and coworkers* have recently published a systematic review to evaluate the scientific literature quality of clinical laboratory quality improvement. The authors assessed 726 papers published from 1981 to 2021 using a *Quality Improvement – Minimum Quality Criteria Set* and found that the average score of QI-MQCS for quality improvement papers from 1981 to 2000 was 2.5, while from 2001–2020, it was 6.8, indicating continuous high-quality improvement in clinical laboratories [10].

26.10 Future Perspectives

Working practices in diagnostic laboratories will change with time. New ways of working will arise and will need more sophisticated forms of leading and cooperation. One example can be seen with the division of activities across multiple working sites, e.g. DNA extraction in a central division, setup and preamplification in another laboratory, finishing with the DNA sequencing in an interdisciplinary laboratory for sequencing and analysis of raw data by bioinformaticians.

There will likely be increasing use of both automation and complex analyzing devices, like NGS, Nanopore, and MALDI-TOF, which will generate much more information about genes, haplotypes, chromosomes, and epigenetics. While in former times single genes had been analyzed, we will see in future screening strategies of a panoply of DNA sequences or SNPs of interest, deployed throughout the human genome.

Information will be too complex to be analyzed on single worksheets, and bioinformatics will become more and more important. Additionally, the influence of artificial intelligence has just started and opens for the next generation of H&I technicians a completely new horizon in data analysis and disease association. This will surely include a new era of supercomputers and algorithms in diagnostics. All these new working practices will evolve rapidly, so laboratories and institutions will need to cooperate with each other.

In future, personalized medicine will become a standard in hematologic diagnostics. HLA- and blood group alleles may be analyzed in parallel [19], and this could also be combined with larger panels of variants to screen, e.g. tumor gene alleles, pharmacogenetics, gene rearrangements, and changes in epigenetic expression patterns. This will lead to a much more targeted treatment with either antibodies or lymphocytes and will prevent patients from unexpected reactions against pharmaceuticals (e.g. as already known for HLA-B*57:01 in HIV care) and will provide a highly sophisticated analysis of tumor cell differentiation and disease staging for a better outcome and patient quality of life.

Having this in mind, we suggest that QM-Systems also have to evolve over time. As seen in the past, newly generated results will first be discussed by scientists and clinicians in small groups, workshops, and scientific societies, leading afterward to the development of standards by professional bodies and again to new national guidelines.

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As a consequence, QM-Systems should not be based on assuring compliance with only a single regulation but should guarantee compliance with all relevant standards or QMS elements.

Looking from other perspectives, the different standards will fulfill different tasks for different purposes, and without competition between them. We should therefore follow the given or newly developed standards as they are needed in routine work, with the understanding of their individual usefulness for the benefit of the patients.

Table of Acronyms and Abbreviations

ASHI	American Society for Histocompatibility and Immunogenetics
CAR-T	Chimeric antigen receptor T-cells
CDC	Complement dependent cytotoxicity
EFI	European Federation for Immunogenetics
ELISA	Enzyme-linked Immunosorbent-Assay
EPT	External Proficiency Testing
FACT	Foundation for the Accreditation of Cellular Therapy
GLP/GMP	Good Laboratory Practice/Good Manufacturing Practice
H&I	Histocompatibility and Immunogenetics
HLA	Human Leukocyte Antigens
HSC	Hematopoietic Stem Cells
HSCT	Hematopoietic Stem Cell Transplantation
IAF	International Accreditation Forum
ILAC	International Laboratory Accreditation Cooperation
ISO	International Organisation for Standardization
JACIE	Joint Accreditation Committee of the International Society for Cell and Gene Therapy (ISCT) & European Society for Blood and Marrow Transplantation (EBMT)
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization-Time of Flight
NAB/NSB	National Accreditation Body/National Standardization Body
NGO	Non-Governmental Organisation
NGS	Next Generation Sequencing
PBSC	Peripheral Blood Stem Cells
PCR	Polymerase Chain Reaction
PDCA	Plan-Do-Check-Act
QMS	Quality Management System
SNP	Single-Nucleotide Polymorphism
SSOP	Typing with Sequence-Specific Oligonucleotide Probes and PCR
SSP	Typing with Sequence-Specific Primers and PCR
Tx	Transplantation
WHO	World Health Organisation
WMDA	World Marrow Donor Association

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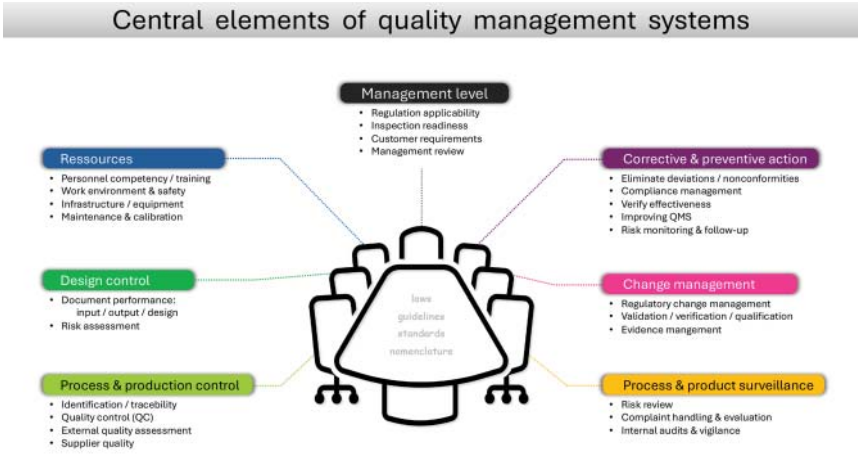
26.A Central Elements of Quality Management Systems



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26.B Comparison Chart of EFI-Standards, ISO 9001, and ISO 15189

		Application of QMS elements in different Quality schemes		
QMS elements	**Description**	**EFI accreditation (reference to Standards 8.1 and Procedure Manual)**	**ISO 15189:2022 (chapter of the norm)**	**ISO 9001:2015 (chapter of the norm)**
Organizational structure	• Mission statement with core purpose and focus	• Not specified	• Not specified	• Yes. (5.2)
	• Strategic plan which defines objectives and how to pursue them	• Not specified	• Yes. (5.5)	• Yes. (6.2)
	• Senior management committed to implementation of QMS (written in a policy)	• Yes. Director must review lab procedure manual (C1.1.2.1)	• Yes (5.4.2)	• Yes. (5.1.1)
	• Organizational structure which defines roles and responsibilities: organogram	• Yes. Requested in application file	• Yes (5.4.1)	• Yes. (5.3)
	• Nomination of a quality manager	• Yes. (B4.2)	• Yes (5.4.2)	• Yes. (5.3)
Personnel	• Number of personnel should be sufficient	• Yes. (B4.3)	• Yes. (6.2.1)	• Yes. (7,1,2)
	• Job descriptions should be available with qualifications	• Yes. (B3, B4)	• Yes. (6.2.2)	• Yes. (7.2)
	• Personnel records should be available	• Not specified • Yes. Competence evaluation and continuous education (B5)	• Yes. (6.2.2) • Yes. (6.2.4)	• Yes. (7.2) • Yes. (7.2)

	• **TRAINING:**
	  • Training program should be designed to allow personnel to acquire and update competence
	  • Training on QMS must be provided
	  • Competency assessment plan
		• Not specified
		• Yes. (B5)
			• Not specified
			• Yes. (6.2.2)	

Risk-based thinking	• The laboratory should have a process to assess different kinds of risk (to patients, business continuity), actions to mitigate risks and opportunities for improvement	• Not specified	• Yes. (5.6 and 8.5.1)	• Yes. (6.1)
Management of processes and procedures	• New procedures and equipment should be validated before use. This also regards computer systems	• Yes. (C1.3.2.1). Computer software (C3.2.2)	• Yes. (7.3.3 and 6.4.3)	• Partially (7.1.3)
	• Documented SOPs (standard Operating Procedures) describing each procedure should be available	• Yes. Laboratory procedure manual (C1.1)	• Yes. (7.3)	• Yes. (7.5.3)
	• SOPs should be up-to-date and regularly reviewed	• Yes. (C1.1.2.1)	• Yes (7.3 and 8.3)	• Yes. (7.5.3)

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QMS elements	Description	Application of QMS elements in different Quality schemes		
		EFI accreditation (reference to Standards 8.1 and Procedure Manual)	ISO 15189:2022 (chapter of the norm)	ISO 9001:2015 (chapter of the norm)
Documentation and record keeping	• Quality documentation should guarantee that the QMS is known throughout the laboratory	• Yes. (C1.1)	• Yes. (8.2.4)	• Yes. (7.5.3, 7.3)
	• Written policies, SOPs or work instructions, worksheets, forms, records, and labels are all important features of the documentation system	• Yes. (C1.1)	• Yes. (8.3)	• Yes. (7.5.3)
	• The laboratory should define who writes the documents, who verifies them and who approves them	• Partially. (C1.1.2.1)	• Yes. (8.3.2)	• Yes. (7.5.2)
	• Records are worksheets/forms on which the activity performed, and the results obtained are documented and stored	• Yes. Records and test results (F3)	• Yes. (8.4.1)	• Yes. (7.5.3)
Facilities	• Laboratories should be built and maintained to be safe for personnel and visitors	• Yes. (C.2)	• Yes. (6.3)	• Yes. (7.1.3)
	• National regulations on safety in the workplace should be followed	• Yes. (C1.4, C2.1.1.1)	• Yes. (6.1-.4)	• Yes (4.1)
Equipment	• Number of equipment should be adequate for workload	• Yes. (C2.2)	• Yes. (6.4)	• Yes. Generic. (7.1.3)
	• Equipment should be used according to manufacturer's instructions	• Not specified.	• Yes. (6.4.4)	• Not specified
	• Equipment should be validated before use	• Yes. (C1.3.2.1)	• Yes. (6.4.3)	• Not specified
	• The laboratory should have a maintenance plan for all the equipment in their inventory, which indicates timeframe for maintenance, cleaning, and calibration	• Yes. (C2.2.1.1)	• Yes. (6.4.5)	• Yes. (7.1.5)
Reagents and consumables	• The laboratory should have an inventory management system for their reagents and consumables	• Yes. (C2.3)	• Yes. (6.6)	• Yes. Generic (8.5.4)
	• Incoming reagents and materials should be evaluated before entering routine	• Yes, in Reagents (E1) and methods (E4)	• Yes. (6.6.2)	• Yes. Generic (8.5.1)
	• The laboratory should keep a list of approved suppliers	• Not specified	• Not specified	• Yes. (8.4.3)
	• Certificates of compliance should be requested from the suppliers	• Not specified	• Yes. (6.6.7)	• Yes. Generic (8.4.3)

(*Continued*)

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QMS elements	Description	Application of QMS elements in different Quality schemes		
		EFI accreditation (reference to Standards 8.1 and Procedure Manual)	ISO 15189:2022 (chapter of the norm)	ISO 9001:2015 (chapter of the norm)
Quality control	• Quality control regards procedures carried out during the performance of the test that provides information on the state of the test • Quality control should test reagents, equipment and methods used in the procedure on order to check that everything is working as expected • SOPs should be written describing quality control, and should include instructions on how to proceed when quality control gives unexpected results	• Yes. Systems for continuous Test Evaluation and Monitoring (C1.3) • Yes. Methods (E4) • Yes. (C1.3)	• Yes. (7.3.7) • Yes. (7.3.7) • Yes. (7.3.7)	• Yes. Generic (8.5.1) • Yes. Generic (8.5.1) • Yes. Generic (8.5.1)
External proficiency testing	• Laboratories should participate in external quality assessments organized by independent providers who send a set of samples of known but undisclosed content and compares results with the performance of other laboratories	• Yes. (D)	• Yes. (7.3.7.3)	• Yes. Generic (9.1.3)
Management of non-conforming product	• Laboratories should have a documented system for dealing with non-conformities (deviations from established standards, specifications, procedures, or requirements) • Personnel should receive training on how to recognize and manage non-conformities	• Yes. (C1.2.2, C1.5) • Not specified.	• Yes. (7.5, 7.7 and 8.7) • Not specified	• Yes. (10.2) • Yes. Generic (7.3)
Monitoring and evaluation of the QMS	• The effectiveness of the QMS should be assessed on a regular basis by: • Quality management reviews: Are performed to monitor the quality system effectiveness and its operations, with the involvement of senior management, and to identify opportunities for continual improvement in the organization	• Yes. • Yes. (C1.2.1.1)	• Yes. • Yes. (8.9)	• Yes. • Yes. (9.3)

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QMS elements	Description	Application of QMS elements in different Quality schemes		
		EFI accreditation (reference to Standards 8.1 and Procedure Manual)	**ISO 15189:2022 (chapter of the norm)**	**ISO 9001:2015 (chapter of the norm)**
	• Internal Audits: performed on a regular basis (usually annually) by trained staff from the organization itself • External Audits: carried out by trained Auditors from outside the organization, for example accreditation agencies	• Not specified • Yes. (A11 and Procedure Manual)	• Yes. (8.8.3) • Yes.	• Yes. (9.2) • Yes. By Certification Body.
	• Organizations are encouraged to collect Performance Indicators, i.e. quantifiable measures to assess the progress of the organization in achieving its planned results against its objectives.	• Not specified	• Yes. (8.8.2)	• Yes. Generic (9.1)
Continuous Quality Improvement	• Continuous quality improvement (CQI) • Continuous quality improvement refers to an ongoing, systematic, and collaborative process aimed at improving the safety, effectiveness, and efficiency of products and services. It involves monitoring and evaluating various aspects of the processes, identifying areas for improvement, implementing changes, and measuring the impact of those changes. The goal of CQI is to ensure that practices consistently meet or exceed established standards and guidelines while adapting to evolving best practices.	• Not specified.	• Yes. (8.6)	• Yes. (10.3)
Technical standards	• Technical standards referring specifically to Histocompatibility and Immunogenetics	Yes. (Section E4–Methods: E4.1 CDC, E4.2 Antibody testing and crossmatching, E4.3 Elisa, E4.4 Flow cytometry, E4.5 Nucleic acid analysis, E4.6 Bead array, E4.7 SSP, E4.8 SSOP,	• No	• No

(*Continued*)

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QMS elements	Description	Application of QMS elements in different Quality schemes		
		EFI accreditation (reference to Standards 8.1 and Procedure Manual)	ISO 15189:2022 (chapter of the norm)	ISO 9001:2015 (chapter of the norm)
		E4.9 Sanger sequencing, E4.10 NGS, E4.11 Hematopoietic chimerism engraftment, E4.12 Analysis of other immunologic markers. Section E5-Clinical applications: E5.1 Renal and/or pancreas transplantation, E5.2 Other organ transplantation, E5.3 Hematopoietic stem cell transplantation, E5.4 Transfusion, E5.5 Disease association. Section F-Post-analysis processes: F1.1 Terminology, F1.2 Phenotypes and genotypes, F1.3 Haplotype assignment, F2 Other immunological markers, F3 Records and test reports.)		

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26.C International Cooperation in Laboratory Accreditation

A) **International Cooperations: ISO Standards and Laboratory Accreditation**

Accreditation is based on two parts:

a) ISO standards

b) ILAC and IAF activity as ***oversight bodies*** and ***governance***

1) ISO = International Standardisation Organisation (or norming organization) [12]
   - ISO is just preparing and providing standards
   - ISO provides robust standards with a small margin for interpretation
   - ISO itself is not doing any assessments
   - 171 National Standardisation Bodies (NSB) are members of ISO

2) IAF = International Accreditation Forum [20]
   - focusses on processes, services, management systems, personnel, validation, verification, and conformity assessment
   - oversight body for quality management
   - Is working very closely together with ILAC (all regions are members of IAF)
   - Is not performing laboratory assessments

3) ILAC = International Laboratory Accreditation Community [21]
   - Is an Oversight Body for Laboratory & Inspection Body Accreditation
   - Is working very closely together with IAF
   - Is solely inspecting National Accreditation Bodies (in 2023 a total of 131)
   - ILAC is organized worldwide in subregions:
     - *EA – European Cooperation of Accreditation*
     - *IAAC – Inter-American Accreditation Cooperation*
     - *APLAC – American-Pacific Laboratory Accreditation Cooperation*
     - *SADCA – South African Development Community Cooperation in Accredt.*
     - *ARAC – Arabic Accreditation Cooperation*
     - *AFRAC – African Accreditation Cooperation*

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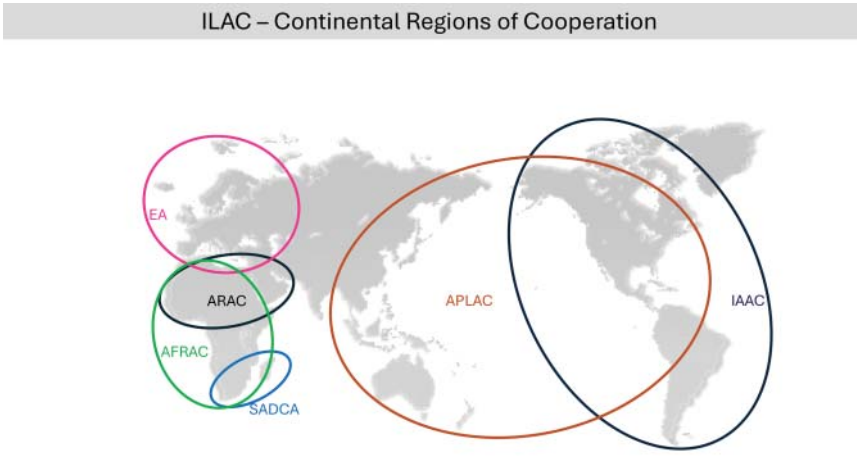
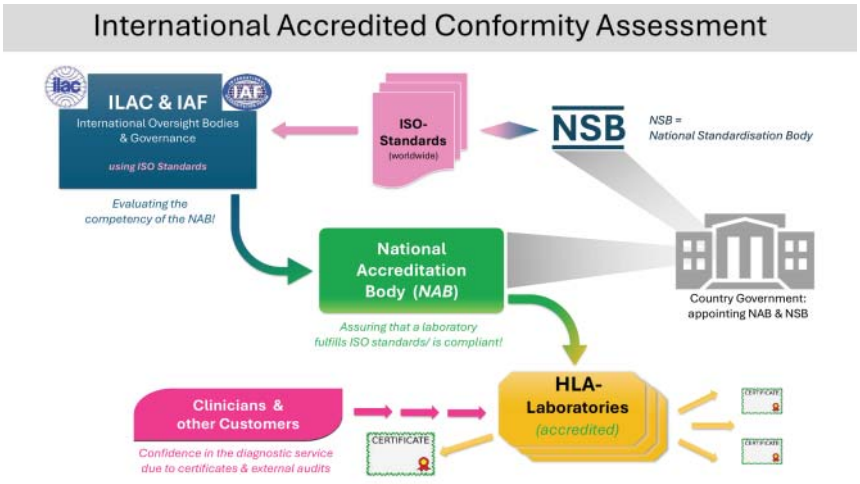


B) **Working principles of IAF/ILAC and governmental appointment of NAB/NSB** [12, 20–21]



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26.D Accreditation, Certification, and Licensing. A Synopsis

	Accreditation	Certification	Licensing
Focus on …	Processes and procedures	Persons or processes	Processes and procedures
Duty?	Voluntary	Voluntary	**In**voluntary, due to special national interest
Provided by …	External organizations/ Accreditation Bodies (NABs)	Professional organizations: Certification Bodies (CBs) or Private Institutions	State government, for the confidence of customers and clients
Given to …	Organizations, agencies, or programs	Individuals, facilities, programs, agencies, or organizations	Individuals or facilities with specialized services
Contents …	Demonstration to clients that an organization meets a rigorous set of standards that are focused on the quality of services and evidence-based practices	Confirmation that an individual or organization meets a minimum set of predetermined and standardized criteria to perform a specific function	Governmental requirements, including laws, guidelines, a rigorous set of standards, and evidence-based practices, with a focus on public safety
Using …	ISO standards, laws, and guidelines	Self-defined or ISO standards, laws, and guidelines	Defined national/ international standards, laws, and guidelines
Recognition/ Acceptance	Accepted worldwide, including national legislation	Accepted by professional bodies and scientific societies, without national legislation	Fully accepted within a state, including national legislation
Typically for …	Public and private medical laboratories in national/international contact	Medical laboratories in national/international contact	Production of blood products or specialized service of public interest

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