Stem Cell Biology and Regenerative Medicine

Paul J. Fairchild *Editor*

The Immunological Barriers to Regenerative Medicine

Humana Press

Stem Cell Biology and Regenerative Medicine

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Humana Press

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This book is dedicated to my wife, Jackie, whose unswerving love, support and encouragement over the past 25 years, remains a constant source of strength and inspiration in a rapidly-changing world

Preface

Few could doubt the need for regenerative medicine. While the increase in life expectancy we have witnessed throughout the developed world over the past 80 years is undoubtedly a medical success story of unprecedented magnitude, the accompanying increase in incidence of non-communicable diseases (NCDs), with a chronic or degenerative aetiology, represents a significant challenge of the twenty-first century. It is estimated, for instance, that the worldwide incidence of mortality due to NCDs will rise to 52 million per year by 2030, while deaths through infectious disease will continue to decline throughout the same period. Such changes in modern healthcare needs, have created an almost insatiable demand for new treatments capable of harnessing the properties of stem cells to replace diseased or effete cell types, or that rejuvenate tissues from within, through the activity of endogenous stem cells. And there have been numerous recent advances that represent significant steps towards the realisation of this vision. While the routine derivation of human embryonic stem cells (hESC) has made pluripotency accessible in man for the first time, the advent of induced pluripotency has paved the way for its clinical application to be tailored to the needs of the individual. Furthermore, preliminary successes in the treatment of diseases such as macular degeneration of the eye through cell replacement therapy suggest that we may at last be on the cusp of reaping the benefits of the past 15 years of research into the nascent field of regenerative medicine.

Nevertheless, fundamental challenges remain to be addressed before such developments may have any significant impact on global health. The British Government's *Forward look in regenerative medicine*, convened in September 2011, identified the immune response directed at stem cell-derived tissues to be a fundamental roadblock to progress. Although the early days of regenerative medicine were accompanied by unfounded optimism that tissues differentiated from hESC or, more recently, induced pluripotent stem cells (iPSC), might prove to be poorly immunogenic, it is now widely accepted that cell therapies pose no fewer immunological challenges than whole organ transplantation: indeed, unlike conventional transplants, the propensity for tumorigenesis of pluripotent stem

cells, suggests that long-term immune suppression is unlikely to offer a solution to rejection in this particular setting.

It is against such a backdrop that this volume offers an analysis of the scale and nature of the immunological issues facing regenerative medicine, drawing on the expertise of laboratories around the world who have taken up the challenge of applying their expertise in immunology to the vagaries of stem cell biology. In Part I, we explore the extent to which the principles of allograft rejection, learned over several decades from our experiences of whole organ transplantation, apply within the unique context of cell replacement therapy. Part II discusses various innovative ways of addressing the issues of immunogenicity, while, in Part III, we focus exclusively on the induction of immunological tolerance through a variety of novel approaches. It is our hope that this systematic analysis of the current state of the field will galvanise efforts to solve an issue which has so far remained intractable.

I am, of course, deeply indebted to all the authors for their patience and commitment to completing this project. Furthermore, there are many who have played an important part in its completion, often in subtle ways, and invariably without realising how important their contributions have been. I have, for instance, been inspired by many friends and colleagues, of which Bébhinn Ramsay, Steve Cobbold and Kathleen Nolan deserve special mention. The members of my laboratory should likewise be singled out, not only for their encouragement and the many scientific insights they have offered, but for the temporary neglect they have endured with such good humour. To this end, I would like to thank Tim Davies, Kate Silk, Alison Leishman, Naoki Ichiryu, Simon Hackett and Patty Sachamitr for their loyalty and for creating such a dynamic and enjoyable environment in which to work. It would be remiss of me not to acknowledge the enormous debt of gratitude I owe my mentors, past and present, for instilling in me their enthusiasm for science and its application to medicine. Jonathan Austyn, David Wraith, Richard Gardner and Herman Waldmann have all invested huge amounts of time and resources in me over the years, often with precious little reward, but their efforts have certainly not been overlooked! Finally, as is so often the case, it is my wife, Jackie, and my son, Richard, who deserve the greatest recognition for their ongoing support and unfaltering love and encouragement: without their sacrifice of holidays and our usual family Christmas, this volume would never have been completed!

Oxford, UK Paul J. Fairchild

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Part I
The Immunogenicity of Stem Cells

Chapter 1
Mechanisms of Immune Rejection of Stem Cell-Derived Tissues: Insights From Organ Transplantation

Eleanor M. Bolton and J. Andrew Bradley

Abstract The use of embryonic, induced pluripotent, or adult stem cells is upheld as a potentially valuable therapeutic approach for replacement or repair of diseased and damaged tissues, partly because these immature cells are considered to be non-immunogenic. It is becoming increasingly clear, however, that tissues differentiated from such stem cell sources have the potential to express immunogenic molecules and will be susceptible to a patient's immune response. This chapter draws on experience of organ and tissue transplantation and the study of transplant immunology to identify cellular and molecular mechanisms that are likely to be relevant to the rejection of stem cell-derived tissues. Pathways of cellular recognition and immune activation are described, together with effector mechanisms that may be responsible, not only for destruction of stem cell transplants, but also for regulating immune responses, thereby improving their chance of survival.

1.1 Introduction

Regenerative medicine is a research discipline whose aim is to establish regeneration, repair or replacement of diseased or damaged tissues, cells and organs, using a variety of approaches. It is anticipated that scientists will learn how to actively and specifically direct the differentiation of stem cells ex vivo toward the recreation of functioning tissues and organs that may be used for repair and

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replacement, and this chapter will consider the immunological implications of those aims. The term stem cells in this context includes pluripotent embryonic stem (ES) cells, induced pluripotent stem cells and adult stem cells, and their sources are, respectively, embryos at very early stages of development, terminally differentiated somatic cells, and stem cells found within adult, functioning tissues, and organs.

The use of stem cells for repair and replacement necessitates some form of tissue culture and manipulation of stem cells followed by their transfer either back into the original cell donor or into an unrelated individual. The transfer of unrelated cells is likely to invoke, to a variable degree, an immune response in the recipient that may culminate in rejection of the transferred tissue.

Several experimental studies have used embryonic or adult stem cells, and differentiated cells derived from these early developmental stages, to treat a range of animal models of human diseases, including heart disease, liver failure, diabetes, and neurodegenerative diseases but the possibility that such interventions may fail because they initiate immunological rejection which has often been overlooked. Many studies using tissue transplanted between outbred rodents or across species have failed to report whether immunosuppression was used. In studies involving transplantation of fetal or ES cell-derived neurological tissue to the brain, recognized to be an immunologically privileged site by virtue of an intact blood–brain barrier, there is a lack of consensus on the need for immunosuppression or a need to use immuno-incompetent recipients. Moreover, many other studies have reported failure of engraftment of fetal or ES cell-derived tissues that is best explained by tissue incompatibility and immunological rejection. To those involved in traditional cell and tissue transplantation this apparent oversight seems surprising as graft rejection has long been recognized as the major barrier to successful transplantation.

To understand the immunological challenges posed by transfer of stem cell-derived tissue, insight may be gained from transplantation of hematopoietic stem cells to treat patients with immune deficiency or blood malignancies. It is also relevant to refer to the extensive body of knowledge of tissue rejection gained from the study of organ transplantation. The historical assumption that stem cells are not immunogenic and therefore not susceptible immune-mediated rejection is now being challenged and many groups are studying the immunogenicity of stem cells and their differentiated progeny. This chapter reviews the immunological basis for rejection of tissue and organ allografts on the basis that many of the principles and lessons learned likely apply also to stem cell transplantation.

1.2 Historical Perspective

Following Landsteiner's discovery of human blood groups in 1900 and the recognition that blood group matching enabled successful blood transfusion, it was perhaps a logical progression to attempt to transplant other tissues between blood group

matched individuals, but early experimental attempts were met with consistent failure. The era of clinical organ transplantation began in the 1950s after the first successful kidney transplant was performed by Joseph Murray and colleagues between genetically identical twins in Boston in 1954 [1]. This and other kidney transplants between identical twins demonstrated clearly that organ transplantation was feasible if the immunological hurdles could be overcome and a search for effective immunosuppressive agents began. Renal transplantation became firmly established as a successful treatment for end-stage renal failure with the introduction of effective immunosuppressive drugs, notably a combination of the 6-mercaptopurine derivative, azathioprine, and corticosteroids [2, 3]. The requirement for immunosuppression was supported by the earlier work of the biologist, Peter Medawar who observed the rejection of skin grafts in burns patients and then went on to study the phenomenon systematically in rabbits. He was the first to show unequivocally that transplant rejection was a manifestation of the immune system recognizing the presence of "foreign", or "non-self" tissue, since skin grafts within the same individual were not rejected while grafts between unrelated individuals were always rejected. Moreover, a second graft from the same donor to a recipient that had rejected a first graft was rejected in accelerated fashion, but when the second graft was from a different unrelated donor it was rejected in normal tempo, demonstrating the development of specific immunological memory [4–6]. An understanding of these observations drew on earlier experimental studies of transfer of malignant tumors between different strains of inbred mice, where the survival or rejection of the transplanted cells was shown to be genetically controlled [7]. Another of Medawar's important contributions was the demonstration of neonatal immunological tolerance: neonatal inbred mice injected with lymphocytes from an unrelated inbred strain were unable, as adults, to reject a skin graft from the same donor strain as the injected cells while they rapidly rejected an unrelated skin graft [8]. Together with the pioneering observations of Medawar, a series of seminal advances over the next two decades provided the basis for current understanding of transplant rejection:

- Frank MacFarlane Burnet proposed the clonal selection theory to explain the development of self-tolerance and the inability to generate self-directed antibody responses [9];
- Gorer and Snell described the genetically determined "histocompatibility complex" antigens that were responsible for rejection of mismatched tissues [10];
- Gowans and colleagues demonstrated a key role for recirculating lymphocytes in both antibody responses to injected soluble antigens, and cell-mediated responses to skin grafts [11];
- Jacques Miller highlighted the importance of thymus-derived T lymphocytes in a range of immunological responses including skin graft rejection, which, together with the earlier observations of Bruce Glick on the role of Bursa-processed cells in antibody responses but not skin graft responses, revealed the dichotomy in the function of lymphocytes [12].

These and other important findings from the 1950s and 1960s established the paradigm of transplantation immunology and provided the basis for immunological

dogma that remains relevant today in the context of both tissue and stem cell transplantation.

1.3 Terms Commonly Used in Transplantation

Several technical terms are used to describe the type and origin of a transplant and to imply its likely outcome (Table 1.1). The early transplantation papers of Medawar and of Murray and colleagues referred to "autografts" and "homotransplants" or homografts. The term "autograft" (or autologous graft) is self explanatory, meaning a transplant of skin, bone marrow, or other tissue within the same individual, and is a term that is still in use. The term "homograft", in contrast, is not a useful term because while it refers to a transplant from one individual to another, it does not distinguish between a transplant from an unrelated donor and from a genetically identical donor. Instead, the terms "allograft" and "syngeneic graft" are used, in both clinical and experimental transplantation, to refer to transplants from non-identical donors and from genetically identical donors (e.g., identical twin), respectively. A xenograft is a transplant from one species to another. Only the terms autograft and syngeneic graft imply that the transplant will not elicit an immune response, and in all other cases of transplantation to a fully immunocompetent recipient, it may be assumed that unless effective immunosuppression is used, the transplant will invariably be rejected because of an immune response against non-self tissue. This applies as much to cellular transplants as to tissues and organ transplants because rejection is initiated by the presence of mismatched histocompatibility antigens that are expressed by virtually all nucleated cells of the body. The challenge, in the case of regenerative medicine, is to determine when, and to what extent, histocompatibility antigens are expressed by ES cells and their differentiated derivatives.

1.4 Tissue Compatibility

The immunological barriers to regenerative medicine are, in principle, the same as those for successful bone marrow, tissue, and organ transplantation. Rejection occurs because of allelic differences between transplant donor and recipient at a number of genetic loci that are included within the ABO blood group system, the major histocompatibility complex (MHC) and the minor histocompatibility (mH) antigens. ABO blood group antigens are expressed at the cell surface, not only of blood erythrocytes but also on most epithelial and endothelial cells. MHC molecules are also expressed at the cell surface, as class I and class II molecules which have variable tissue distribution reflecting their immunological function. Both ABO and MHC tissue antigens are, therefore, easily recognized by the immune system and may elicit powerful immune responses resulting in rapid graft rejection. mH antigens are allelic

Table 1.1 Terms in transplantation immunology

Term	Explanation
Allograft	Transplantation of tissue or organ between genetically dis-similar individuals
Syngeneic graft	Transplantation between genetically identical individuals
Autograft	Transplantation of tissue within one individual
Xenograft	Transplant from one species to another
Privileged site	An anatomical site, e.g., the anterior chamber of the eye, where transplanted tissue is protected from graft rejection
MHC	Major histocompatibility complex: the conserved gene region encoding highly polymorphic class I and class II cell surface molecules that present antigenic peptides to T lymphocytes
mH	Minor histocompatibility antigens: polymorphic intracellular proteins that, when presented as peptides, may contribute to immunological rejection
HLA complex	Human leukocyte antigen complex: term for the human MHC, located on chromosome 6
H-2 complex	Histocompatibility-2: term for the mouse MHC, located on chromosome 17

forms of intracellular proteins and are presented only as antigenic peptides; they are less easily recognized by the immune system but may contribute to, or in certain circumstances be responsible for, graft rejection.

1.4.1 The ABO System

Among cellular transplant procedures, blood transfusion is the most common and ABO blood group compatibility is necessary to ensure safe and successful transfusion. ABO antigens are protein–carbohydrate molecules, termed H antigen, inserted in the cell membrane of erythrocytes. The H antigen locus has three allelic forms that encode the terminal carbohydrate chain of the A antigen form, the B antigen form, or unchanged H antigen, designated O. All individuals have naturally occurring, circulating antibodies of the IgM class with specificity for the non-expressed A or B antigens, that develop during infancy as a cross-reaction response to bacteria colonizing the gastrointestinal tract and expressing similar surface antigens. Thus, blood group A individuals have circulating anti-B antibodies, blood group B individuals have circulating anti-A antibodies, blood group O individuals have both anti-A and anti-B antibodies while those who are blood group AB have no circulating antibodies against ABO antigens. Pre-existing IgM antibodies against ABO antigens rapidly bind to their target molecules on transfused blood or transplanted tissues, activating the complement cascade and the coagulation response, and thereby causing blood lysis and extensive tissue damage.

Since ABO antigens are expressed on many cell types other than erythrocytes, ensuring ABO compatibility is a prerequisite to bone marrow and organ

transplantation. It has recently been shown that ABO antigens are also expressed by both ES cells and by their in vitro-differentiated derivatives such as cardiomyocyte- and hepatocyte-like cells [13], suggesting that ABO matching will be necessary for regenerative medicine.

The Rhesus blood group antigens are another system of erythrocyte-expressed molecules that may elicit a strong but limited antibody response following transfusion of Rhesus-positive blood into a Rhesus-negative individual, but there are no pre-existing anti-Rhesus antibodies and it is not considered necessary to match for Rhesus antigens in tissue or organ transplantation.

1.4.2 The Major Histocompatibility Complex

The MHC is a system of around 200 genes located on the short arm of chromosome 6 in humans (at 6p21.1–21.3) and encoding, among others, three major classes of molecules, two of which have multiple allelic forms (Fig. 1.1 and Table 1.2). These gene products are collectively called Human Leukocyte Antigens, or HLA, because the molecules were originally known to be present on leukocytes but have since been shown to be widely expressed throughout the body [14, 15]. HLA class I and class II molecules have a key role in immune surveillance since their function is to present peptides derived from either newly generated intracellular proteins (including viral proteins) or proteins sampled from the extracellular environment, for presentation to T lymphocytes. Depending on the nature of the peptide, T cells will either be responsive or anergic. The HLA system is the most highly polymorphic gene system in the body; it includes 3 highly polymorphic class I genes whose allelic forms of α-chains combine with the non-polymorphic β2-microglobulin chain to form the heterodimeric class I molecules HLA-A, HLA-B, and HLA-C which are widely expressed in the cell membranes of most nucleated cells in the body. The HLA system also includes three pairs of polymorphic class II α- and β-chain genes whose gene products combine to form the heterodimeric HLA-DR, -DP, and -DQ class II molecules inserted in the cell membranes of specialized leukocytes collectively termed antigen presenting cells (APC), as well as endothelial cells, and certain types of epithelial cells. Their distribution is much less widespread than that of class I molecules. Expression of HLA class I and class II may be both highly upregulated and induced in the presence of pro-inflammatory cytokines, particularly interferon-γ. The HLA system also encodes other, relatively non-polymorphic class I molecules whose tissue distribution is restricted, such as HLA-E and HLA-G which function as recognition elements for cells of the innate immune system, including natural killer (NK) cells. NK cells typically kill cells that express no, or low, classical HLA class I and are facilitated to recognize absence of classical class I by the presence of non-classical class I. Thus, during pregnancy, the trophoblast does not express classical HLA class I, to protect the semi-allogeneic fetus from immune attack, but it does express high levels of HLA-G that engage with NK cell receptors and protect the

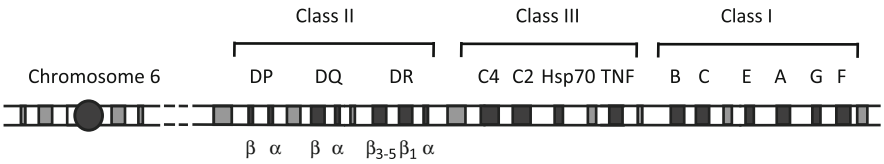

Fig. 1.1 The HLA gene complex. The HLA class I and class II membrane molecules, together with soluble inflammatory proteins (class III), are encoded by a set of genes located on the short arm of chromosome 6, at 6p21.1–21.3

Table 1.2 The HLA gene complex

HLA class	HLA locus	HLA alleles	HLA serological specificities
Class I	A	>1000	28
Class I	B	>1600	60
Class I	C	>650	10
Class II	DRα	3	24
	DRβ1	>750	
	DRβ3-5	>70	
Class II	DPα	>25	6
	DPβ	>135	
Class II	DQα	>30	9
	DQβ	>100	

The HLA class I and class II membrane molecules, together with soluble inflammatory proteins (class III), are encoded by a set of genes located on the short arm of chromosome 6. According to recent data, a total of 3296 HLA-A, -B and -C α-chain alleles encode 2520 proteins, of which 98 are recognized as distinct class I molecules by anti-HLA antibodies [18]. Similarly, 1222 α- and β-chain alleles encode 931 class II molecules of which 39 are recognized by specific antibodies

trophoblast from attack. Other relatively non-polymorphic class II genes encode various proteins involved in antigen processing and presentation, and include the proteasome component LMP genes, the class I-peptide complex assembly genes for TAP1, TAP2 and tapasin, and the class II-peptide complex assembly genes for DM and DO. HLA class III genes encode components of the complement system and certain inflammatory proteins.

As well as having a functional role in antigen presentation, HLA molecules serve as recognition elements for immune cells surveying the body. Because immune cells recognize specific peptides only when presented by APCs bearing MHC molecules identical to those expressed by the lymphocytes themselves, they are able to distinguish different peptides and different MHC molecules—a function termed MHC restriction [16]. However, they are also able to respond to non-self classical MHC molecules in a response that is unique to transplantation, in that a rejection response is initiated in an attempt to destroy the transplanted tissue. For this reason, transplantation usually requires that, where possible, donor and recipient HLA types are closely matched in order not only to minimize the amount of immunosuppression administered, but also to prevent rejection of the transplant and to reduce the risk of graft versus host disease. It is likely that HLA matching

would be advantageous for stem cell transplantation since at some point, HLA molecules will be expressed by the differentiated progeny which may then become targets of a rejection response.

1.4.3 HLA Matching

HLA matching, while desirable, is not a simple matter. There are currently more than 3000 known HLA-A, -B, and -C class I α-chain alleles and more than 1000 HLA class II α- and β-chain alleles, expressed as >2500 distinct class I molecules and >900 class II molecules, although there are only around 140 distinct epitopes recognized by individual antibodies [17, 18]. In a transplant setting, all of these distinct proteins may be antigenic since they are readily accessible to T and B lymphocyte receptors. Moreover, since they are expressed on fetal tissues and on blood cells, any potential transplant recipients that have been pregnant or had a blood transfusion may have become sensitized to non-self HLA molecules and will have generated memory T cells and possibly also circulating anti-HLA antibodies and memory B cells.

Each individual inherits their complement of two HLA alleles at each genetic locus within a section of chromosome inherited from each parent; they will express one allele each of HLA-A, HLA-B, and HLA-C classical class I molecules from each parent and one allele each of the three principal class II molecules (HLA-DR, -DP, and -DQ). Alleles are expressed co-dominantly with little or no crossover within the HLA complex (Fig. 1.2).

In the case of deceased donor kidney transplantation, the HLA tissue type of the deceased donor and all potential recipients is determined; for each donor, attempts are then made to select recipients from the transplant waiting list that are well-matched for HLA-A, -B, and -DR locus antigens. Such matching confers a survival advantage for the transplant by minimizing the risk of rejection and reducing the burden of immunosuppression. A further advantage of a kidney graft that is well matched for HLA is that, should the graft subsequently fail, it is less likely the recipient will develop anti-HLA antibodies that might rule out a second transplant. A cross match test is also performed on the selected kidney donor–recipient pair to exclude the possibility of rapid or hyperacute rejection resulting from existing circulating anti-donor HLA antibodies. For bone marrow transplantation (or hematopoietic stem cell transplantation) where the donor is not an HLA-identical sibling, HLA matching requirements are more stringent. The aim is to achieve a match at the HLA-A, -B, -C, -DR, and -DQ loci, not only to minimize the risks of rejection of the transplant but also to reduce the chance that the immune cells that constitute the transplant may themselves recognize the host as foreign and give rise to graft versus host disease.

For hematopoietic stem cell transplantation and for renal transplantation the benefits of HLA matching have long been known and remain in spite of improvements in immunosuppression (Fig. 1.3). In the case of other types of solid organ

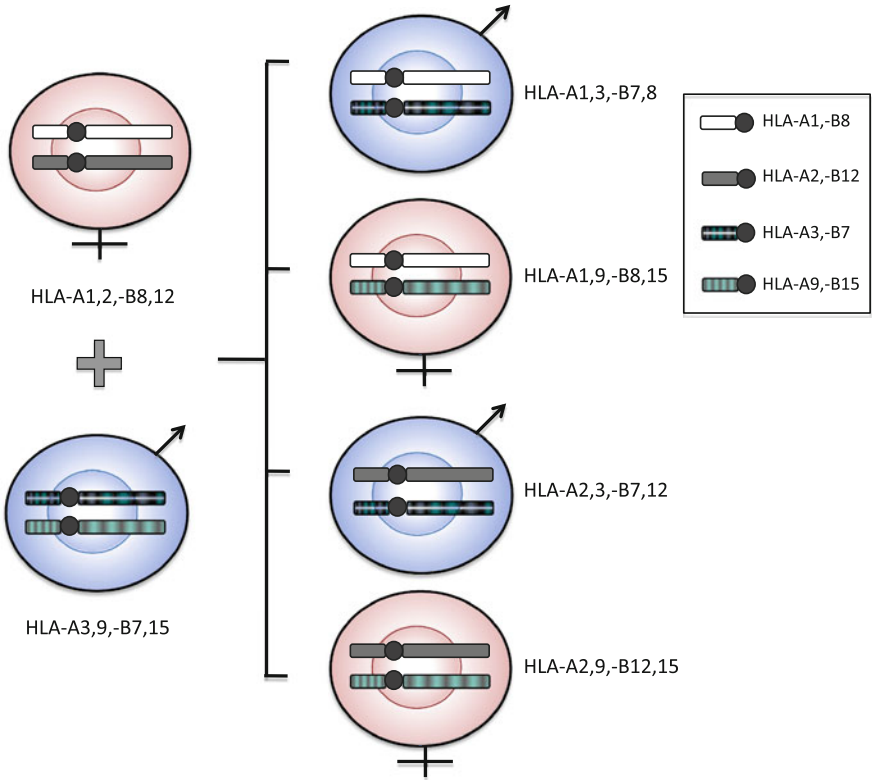

Fig. 1.2 Inheritance of HLA. An individual inherits one copy (haplotype) of the full complement of MHC genes from each parent, and expresses them co-dominantly. Chromosomal cross-over within the MHC is rare. There is a 50 % chance that an individual will have a 1-haplotype HLA match with a sibling, a 25 % chance of a 2-haplotype match, and a 25 % chance of a 2-haplotype mismatch

transplantation, including heart, lungs, and liver, HLA matching is not usually undertaken because any potential advantage of HLA matching is outweighed by the logistic difficulties of finding a well-matched organ for the smaller pool of recipients, the need to consider other factors such as size matching when allocating such organs, and the need to transplant such life-saving organs more promptly before their function is impaired by excessive cold ischemia during storage and transport.

1.4.4 Minor Histocompatibility Antigens

Minor histocompatibility antigens are protein molecules, usually with allelic variants, that are encoded by genes outside of the MHC and take the form of intracellular, rather than membrane proteins. Because of their intracellular distribution they are not

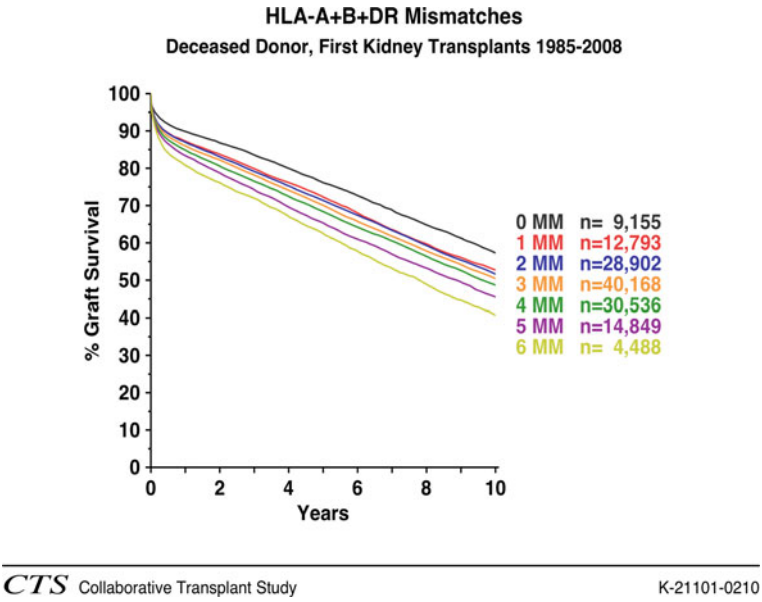

Fig. 1.3 Effect of HLA matching on outcome of renal transplants. Kaplan–Meier plot of kidney graft survival according to number of HLA mismatches (MM) between donor and recipient (where 0 MM represents a full match at each of the two HLA-A, two HLA-B, and two HLA-DR loci, and 6 MM represents expression of different alleles at each of the 6 HLA loci) demonstrating the beneficial effect of HLA matching. Data from the Collaborative Transplant Study (www.ctstransplant.org) reproduced with a kind permission from Professor Gerhard Opelz, University of Heidelberg. This color image is reproduced in grayscale; the lines of the graph are in the same order as the key, with the top line representing 0 MM and the bottom line representing 6 MM

recognized as intact proteins but rather as peptide fragments in the context of MHC. A well-known example of a mH molecule is the male-specific H-Y antigen which, in mice, is capable of causing rejection of male tissue transplanted to a female recipient [19]. An important source of genetic variation in ES cell lines generated from embryos created by nuclear transfer, is mitochondrial gene products, which provide another example of mH antigens. Characteristically, mH antigens contribute to rejection but at a slower tempo when compared with MHC antigens. Clinically, no attempt is made to match for mH antigens prior to transplantation but it is clear from HLA-matched hematopoietic stem cell transplant patients that there remains a requirement for immunosuppression to counteract rejection induced by minor antigen mismatches.

1.5 HLA Structure and Function

The discovery of MHC molecules and their genetic diversity arose from tumor transplantation experiments in mice, where it became clear that blood lymphocytes could recognize and proliferate in response to exposure to non-self MHC

molecules expressed on the cells of genetically unrelated mice. It was puzzling that a system of highly visible, highly polymorphic molecules should exist and that there was a need for their specific recognition by cells of the immune system and by antibodies. During the 1970s it was shown that the function of MHC molecules was to serve as a recognition element for responding lymphocytes, not alone but as a complex with antigenic molecules representing foreign proteins and pathogens. The paradigm of MHC restriction (described by Zinkernagel and Doherty, [16]) was developed from the finding that a clone of T lymphocytes generated by immunizing a strain A mouse with protein X would recognize and respond (by proliferating) to cells expressing strain A MHC complexed with peptide X, but not to strain B MHC complexed with peptide X, nor to strain A MHC complexed with peptide Y. The use of crystallography to reveal the structure of the HLA-A2 molecule, in a landmark paper by Björkman and colleagues in 1987, clarified both the detailed structure of HLA class I molecules and how structure defined their function [20]. The subsequent publication of the structure of class II molecules and T cell receptors (TCRs) completed the picture and provided an understanding of the basis of an immune response: lymphocyte interactions with peptide-MHC complexes [21, 22].

1.5.1 HLA Structure

HLA class I molecules consist of two polypeptide chains of unequal size (Fig. 1.4). The extracellular region of the heavy chain, or α chain has approximately 300 amino acids arranged in three "domains", and includes a transmembrane region as well as a short intracytoplasmic tail. The heavy chain is bound non-covalently to the invariant light chain (*β*-microglobulin) that does not have a transmembrane region. The two distal α1 and α2 domains form the antigen-binding part of the class I molecule, while the membrane-proximal α3 domain has an invariant region that binds weakly to the CD8α molecule during interaction with $CD8^+$ T cells. The α1 and α2 domains each have an area of *β*-pleated sheet surmounted by an α-helical region which together form a peptide binding cleft into which a peptide of around 9 amino acids is inserted. The structure of these two domains is such that the α-helices "present" antigenic peptide for recognition by the antigen-binding regions on the α and *β* chains of the TCR.

HLA class II molecules have been shown, by crystallography studies, to have a similar overall structure to that of class I molecules. Class II molecules have two similar sized, non-covalently bound polypeptide chains, termed α and *β*, each consisting of two extracellular domains, a transmembrane region and a cytoplasmic tail. The distal domain of each chain (the α1 and *β*1 domains) together form a structure that closely resembles the α1 and α2 domain structure of the class I molecule: each of the α1 and *β*1 domains has a region of *β*-pleated sheet surmounted by an α-helical region which together form a peptide binding cleft. The cleft of class II molecules is a more open-ended structure and, typically,

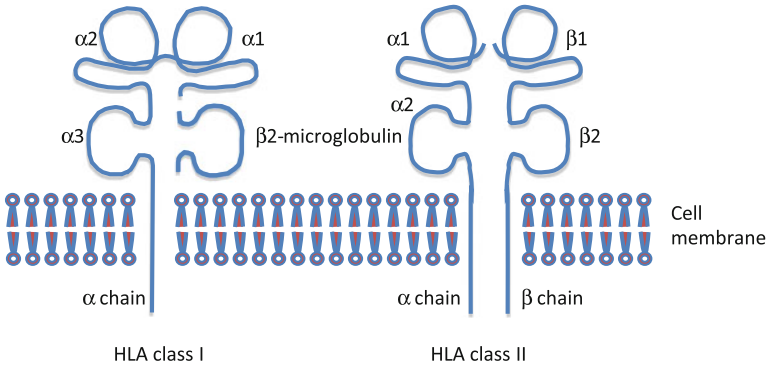

Fig. 1.4 Diagram of the structure of HLA class I and class II molecules. In both molecules, the membrane-proximal, immunoglobulin-like domains have relatively conserved amino acid sequences and provide a site for binding of the accessory CD8 and CD4 molecules, respectively, to strengthen the interaction between T cell and APC. The two distal domains of each molecule have highly polymorphic regions to ensure presentation of a wide range of peptides to the TCR

peptides of around 13 amino acids are presented to the TCR, although peptides can be much longer and have a looped conformation in the cleft. The $\alpha 2$ and $\beta 2$ domains are relatively non-polymorphic and a region of hydrophobic amino acids on each domain where they are closely approximated forms a crevice that is the site for interaction with the CD4 molecule on T lymphocytes.

1.5.2 HLA Function

Extensive gene polymorphism is critical to the function of MHC (or HLA) molecules. Both the β-pleated sheets and the α-helices of both class I and class II molecules have highly polymorphic regions. The resulting variability in amino acid sequences permits diversity of both the peptide binding elements and of the recognition elements presented to the TCR, thereby ensuring that any pathogen encountered is accessible to the immune system. This clearly gives a survival advantage to the species or strain with greatest diversity but is not helpful for regenerative medicine and transplantation.

HLA class I and class II molecules have different cellular distribution which reflects their function. Class I molecules are widely expressed on most nucleated cells throughout the body and their function is to protect the individual from intracellular pathogens such as viruses that replicate by using the host cell replication machinery. Intracellular proteins and peptides are normally packaged for presentation by class I molecules at the cell surface where they can be sampled by $CD8^+$ cytotoxic T cells (Fig. 1.5); as new viral particles are produced their peptides are transported by class I molecules to the cell surface where they are recognized by cytotoxic T cells as foreign, and they respond by killing the infected

target cells. In contrast, the function of class II molecules is to present peptides derived from extracellular proteins and pathogens. Extracellular material is sampled by phagocytosis, or macropinocytosis, or in the case of B lymphocytes, by receptor-mediated endocytosis using the specific B cell receptor or surface immunoglobulin (Fig. 1.5). The resulting membrane-bound vesicles containing potentially dangerous material become increasingly acidic, a process which helps to break down the contents. Endosomes then fuse with lysosomes that break down the contents further into peptides, which are then able to bind to class II molecules and the complex is delivered to the cell surface for presentation to CD4 T lymphocytes. Only specialized APCs, including dendritic cells, macrophages, and B lymphocytes are able to process extracellular material in this way and therefore they are the principal cell types that express class II molecules.

1.6 Induction of the Innate and Adaptive Immune Responses

Expression of MHC molecules is integral to the good health and survival of the species and it may, therefore, be assumed that at some stage in its life cycle, every nucleated cell will express class I molecules, if not class II molecules as well. T cell recognition of MHC-peptide is the first step toward raising an immune response against a potentially dangerous pathogen, and following transplantation, T cell recognition of non-self MHC (expressed on the donor tissue) initiates a rejection response.

The first stage in an adaptive immune response is recognition by $CD4^+$ T cells of an HLA class II-peptide complex. Unless this is a transplant situation, the $CD4^+$ T cell will recognize HLA class II as self, and the peptide as either derived from self-protein, in which case the T cell will normally be tolerant of it, or as foreign peptide, in which case the T cell will become activated. The $CD4^+$ T cell then functions as a helper cell and secretes cytokines that potentially co-ordinate the activation of the entire repertoire of the immune system, termed the adaptive immune response (or acquired immunity). Naïve $CD8^+$ T cells and B lymphocytes differentiate into cytotoxic cells and plasma cells, respectively, but only if they first receive help from activated $CD4^+$ T cells. At the same time, the innate immune response is activated by a range of different stimuli and this system contributes to adaptive immunity [23]. As the response progresses, the adaptive immune system develops specific memory of that particular antigen and if the antigen is encountered at a future date, the resulting immune response will draw on its immunological memory and will respond both more quickly and with greater magnitude. The characteristic features of adaptive immunity are specificity and memory, which are largely absent from innate immunity.

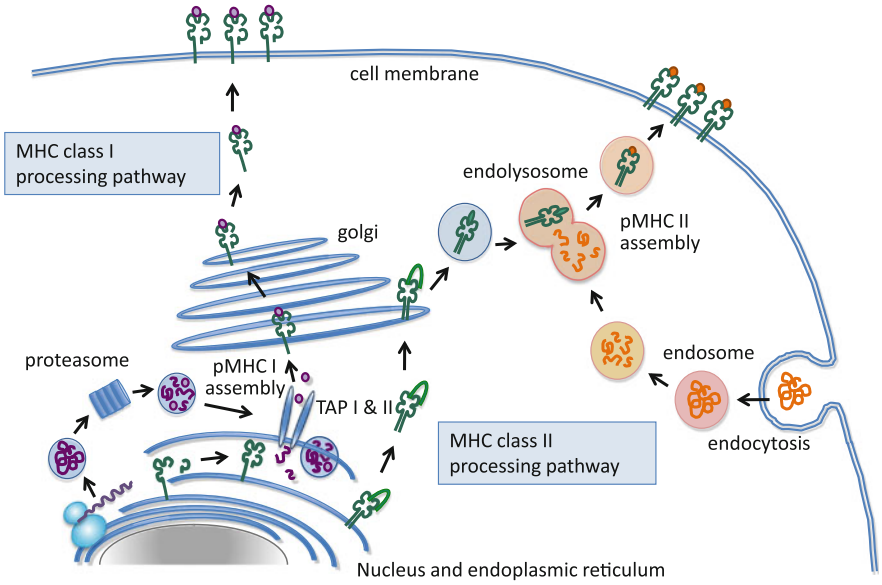

Fig. 1.5 Pathways of antigen processing and presentation. Newly synthesized endogenous proteins (including viral components) are processed and presented by MHC class I molecules, while extracellular proteins are taken up by endocytosis and processed and presented primarily by MHC class II molecules. New protein synthesis occurs when mRNA attaches to a ribosome and the ribosome attaches to ER. In order to maintain a healthy turnover of proteins, ubiquitin-tagged proteins (both normal and mis-folded) are degraded by proteasomes in the cytosol, and further degraded to peptides in the heterodimeric TAP (Transporter associated with antigen processing) molecules located in the ER. MHC class I molecules are simultaneously synthesized at the ER and the correct folding of the heavy chain with β2-microglobulin is stabilized by calnexin. Calnexin is replaced by the class I chaperone proteins, calreticulin, and tapasin, that mediate assembly of the class I molecule with peptide emerging from the TAP molecule. The free MHC class I-peptide complex is transported via the Golgi apparatus to the cell surface where it is embedded in the cell membrane for presentation to $CD8^+$ T cells. MHC class II molecules are synthesised at the ER where the two chains are complexed with the "invariant chain". This complex passes through the Golgi apparatus to be released in lysosomal vacuoles within the cytosol, where the invariant chain is shortened to become the CLIP (Class II associated invariant chain peptide). At the same time, extracellular proteins taken up by endocytosis and enclosed within endosomes are degraded to peptides as the vacuolar pH is reduced. Endosomes and lysosomes eventually fuse and the class II-region HLA-DM molecule facilitates exchange of the CLIP for antigenic peptide to form the MHC class II-peptide complex (a process that may be inhibited, instead, by the HLA-DO molecule). This complex is transported and inserted within the cell membrane for presentation to $CD4^+$ T cells. Two additional pathways, termed autophagy and cross-presentation, enable presentation of endogenous (viral) proteins by MHC class II and exogenous proteins (engulfed, virus-infected dead cells, for example) by MHC class I molecules. These are strictly regulated pathways but are important for provision of help initially for maturation of anti-viral cytotoxic T cells when viruses infect stromal cells that are not professional APCs and therefore lack co-stimulatory molecules. There is evidence that cross-presentation may occur in processing and presentation of alloantigens following transplantation [89]

1.6.1 Innate Immunity

The process of transplantation is inevitably associated with tissue damage through surgery, exposure to potentially infectious agents, and ischemia (cessation of blood supply) followed by reperfusion, all of which are powerful triggers of innate immunity [24] (Fig. 1.6). The production of free radicals, or reactive oxygen species (ROS), is characteristic of ischemic tissue damage followed by reperfusion and is a potent inducer of apoptosis via induction of caspases such as caspase 3. Production of ROS may also be induced by factors in the transplant recipient, including hypertension, hyperlipidemia, viral infections, and immunosuppressive drug toxicity [23]. Tissue damage also induces the production of heat shock proteins and other cellular proteins whose function is to scavenge harmful molecules like ROS. These scavenger proteins express simple repeating molecular patterns termed *damage-associated molecular patterns* or DAMPs that are recognized by receptors termed *Toll-like receptors* (TLRs) expressed by macrophages, neutrophils, NK cells, and dendritic cells [25, 26]. Another important trigger of innate immunity is the introduction of infectious agents where components of bacterial cell walls termed *pattern-associated molecular patterns* (or PAMPs), and single-stranded viral RNA nucleoside components, are recognized by additional members of the family of TLRs expressed by non-specific inflammatory immune cells [27, 28]. The resulting inflammatory environment activates dendritic cells to initiate antigen uptake, processing and presentation, recruits more inflammatory cells via induction of chemokines that regulate cell migration, enhances vascular permeability to encourage drainage of extracellular fluid and free soluble antigen to the draining lymph nodes, and also assists in upregulation of HLA class I and II expression. There is a considerable redundancy of TLR signaling and adaptor protein molecules in the innate response, and they play multiple roles in alloimmunity as illustrated, for example, by studies in TLR-knockout mice demonstrating a critical contribution of the innate response to acute allograft rejection, and maintenance of tolerance (abrogated by administration of TLR ligands) [29–31].

1.6.2 Adaptive Immunity

T cells residing in lymph nodes draining the site of an organ transplant encounter activated donor dendritic cells that migrate out of the transplant when blood circulation is restored, as well as recipient dendritic cells that are able to process and present donor material, such as necrotic cells, shed from the transplant. At this point, the adaptive immune response is initiated as naïve T cells engage with HLA molecules expressed by dendritic cells. Migration of T cells and dendritic cells is critical to the development of adaptive immunity and is mediated by chemokine/chemokine receptor interaction and by integrins. Chemokines are small proteins

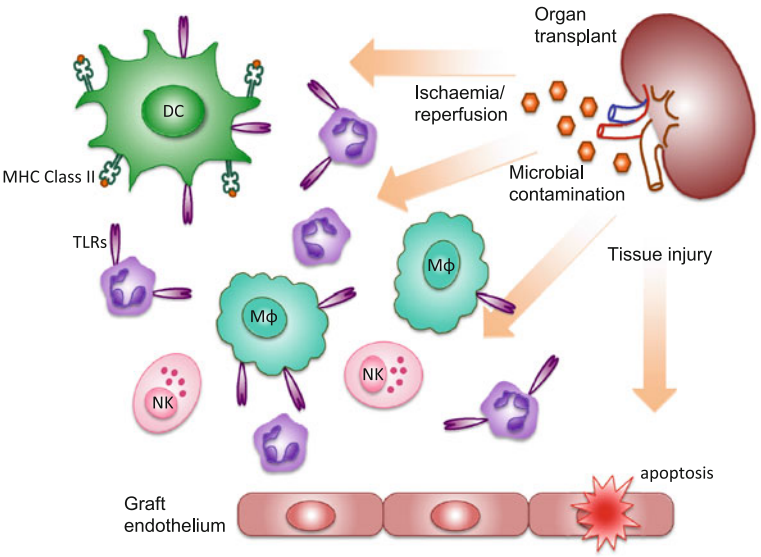

Fig. 1.6 The innate immune response. The process of organ transplantation introduces several triggers of innate immunity, including trauma and tissue damage, ischemia and reperfusion injury, and microbial contamination (viral particles are represented in the figure). Cells of the innate immune system, including dendritic cells, neutrophils, NK cells, and macrophages, express TLRs that engage with a range of molecules such as heat shock proteins released during ischemia/reperfusion injury and donor tissue injury, as well as with pathogen-associated molecular patterns (PAMPs) expressed by microbial contaminants. ROS induced by ischemia/reperfusion injury cause endothelial cell activation and apoptosis, while TLR signaling induces secretion of inflammatory proteins and activates dendritic cells, thereby initiating a link with the adaptive immune (rejection) response

with broad overall similarity that are categorized according to the structural arrangements of cysteine residues that assist in their tertiary folding [32]. Their function is to direct cell migration and they are key mediators of a range of responses involving migration, including immunity, inflammation, homeostasis, wound healing, and angiogenesis. They are produced by a wide range of cell types, including leukocytes and parenchymal cells, following a stimulus, such as viral infection, oncogenesis, and ischemia. Naive T lymphocytes express a set of chemokine receptors (particularly CCR7) that are responsive to concentration gradients of certain chemokines (particularly CCL21) produced by activated macrophages and dendritic cells within secondary lymphoid tissues. This response initiates interaction between naive T cells and APCs, and following antigen recognition, T cells express different chemokine receptors that assist their migration to appropriate areas of the lymphoid tissue where they mature, proliferate, and interact with B lymphocytes that also mature into antibody-producing cells. The contribution of organized secondary lymphoid tissue is critical to the development of an effective rejection response, as demonstrated by the diminished ability of mice lacking secondary lymphoid tissue to acutely reject an organ allograft [33]. In the presence

of an ongoing, chronic rejection response (and in chronic inflammatory autoimmune disease), however, there is evidence for lymphoid neogenesis as accumulations of lymphocytes and dendritic cells may form organized tertiary lymphoid structures within the transplant (or inflamed tissue) that may contribute to a persistent immune response [34, 35].

Lymphocytes that have encountered alloantigen presented by dendritic cells in organized lymphoid tissue are then able to respond in a chemotactic manner to chemokines produced at a distant site of inflammation, or immune stimulus. Their passage through endothelial layers into parenchymal tissue is assisted by a chemokine-induced conformational change in different integrins or adhesion molecules, expressed by both lymphocytes and endothelial cells, which permits their interaction and thereby regulates rolling of lymphocytes along endothelium, arrest, adherence, and transmigration both between and through endothelial cells to the extracellular matrix of parenchymal tissue (Fig. 1.7). Several studies have examined the contribution of chemokines and their receptors to allograft rejection and it is clear that certain interactions play a significant role under defined conditions in the outcome of experimental and clinical transplants, but also that there is considerable functional overlap between these molecules [36].

1.6.3 Natural Cytotoxicity

An important component of innate immunity is contributed by NK cells that are triggered to lyse cells expressing no, or low levels of, classical MHC class I antigens, irrespective of whether they are of autologous or allogeneic origin [37]. They have potent cytolytic activity and secrete a range of cytokines, thereby playing an important role in inflammation and regulation of adaptive immunity. NK cell activity is highly regulated via two sets of receptors:

- inhibitory killer cell immunoglobulin-like receptors (KIRs) in humans and Ly49 receptors in mice, that are induced by immuno-receptor tyrosine-based inhibitory motifs (ITIMs) on classical MHC class I molecules, and NKG2A/CD94 receptors that recognize certain non-classical MHC class I molecules (e.g., HLA-E);
- activatory or "natural cytotoxicity" receptors, including (among others) NKG2D, a transmembrane, lectin-like receptor that recognizes numerous ligands all allied to MHC class I proteins, and including MHC class I chain-related protein A (MICA) and B (MICB) which are expressed as a result of target cell stress.

NK cell activity is induced by cells that are transformed during oncogenesis and viral infection, both of which result in upregulation of NKG2D receptors and downregulation of MHC class I expression. NK cells perform an important function in hematopoietic stem cell transplantation for leukemia therapy following recipient bone marrow ablation, since donor NK cells are able to target any remaining leukemic cells, a response known as the graft versus leukemia effect [38].