GENERAL INTRODUCTION
Chapter 1

CONTENTS

1. INTRODUCTION

2. HISTORY OF BLOOD TRANSFUSIONS

3. THE HUMAN IMMUNE SYSTEM
   3.1. The immune response
       3.1.1. Innate immunity
       3.1.2. Acquired immunity
   3.2. Human leukocyte antigens
       3.2.1. HLA class I molecules
       3.2.2. HLA class II molecules
   3.3. The activation of T cells
       3.3.1. Direct versus indirect allorecognition
       3.3.2. Co-stimulation
   3.4. Effector mechanisms
       3.4.1. T cells
       3.4.2. B cells
   3.5. Immune regulation

4. CLINICAL CONSEQUENCES OF EXPOSURE TO FOREIGN HLA MOLECULES
   4.1. Blood transfusion
       4.1.1. Immune activation
       4.1.2. Transfusion-related immunomodulation
       4.1.3. Leukocyte-depletion
   4.2. Transplantation
       4.2.1. Transplant rejection
       4.2.2. HLA matching and immunosuppressive drugs

5. IMMUNOMODULATION BY PRETRANSPLANT BLOOD TRANSFUSIONS
   5.1. The blood transfusion effect
   5.2. Immunomodulatory mechanisms

6. AIM OF THIS THESIS
1. INTRODUCTION

Blood transfusion is the process of transferring blood or blood components from one individual into the circulatory system of another. Blood transfusions can be life-saving after massive blood loss during trauma or surgery or can be used to treat chronic blood diseases such as anemia and thrombocytopenia. Unfortunately, these therapeutic effects can be accompanied by transfusion complications that involve the recipient’s immune system. Since the immune system is educated to distinguish self from non-self molecules (1), non-self molecules present in the blood product can activate the recipient’s immune system.

Besides immune activation, blood transfusions can modulate or suppress the recipient’s immune system resulting in tolerance. Already in 1953, Billingham, Brent and Medawar showed that infusion of donor cells in a newborn mouse induces lifelong immunological tolerance in a proportion of animals towards the donors’ organs (2). Twenty years later, an enhanced graft survival was observed in kidney transplant recipients who had received pretransplant blood transfusions (3). During the last decades, the beneficial effects of pretransplant blood transfusions are overshadowed by other approaches to improve graft survival (4). Better patient care, compatibility between patient and organ donor and in particular the use of new generation immunosuppressive drugs all positively affect transplantation outcome. However, transplant rejection and health problems due to side effects of immunosuppressive drugs remain a problem and there is still need for strategies that induce a state of tolerance in the recipient. In this thesis we questioned whether a pretransplant blood transfusion may be part of such a strategy in patients that undergo simultaneous pancreas-kidney transplantation (SPKT).

After a short description of the history of blood transfusions and some general immunological aspects, this introduction will give an overview of the immunological and clinical effects of pretransplant blood transfusions.

2. HISTORY OF BLOOD TRANSFUSIONS

In the early 17th century, attempts were made to transfuse humans with animal blood but this resulted in severe complications and high mortality rates. Blood transfusions were abandoned or even prohibited in some countries, until 1816 when John Leacock and James Blundell established that for successful blood transfusion donor and recipient must be of the same species (5). The occurrence of severe transfusion-related complications in the majority of patients urged intensive research on blood transfusion again. In 1900 and 1901, Landsteiner discovered the ABO erythrocyte blood group system. After mixing blood from two individuals, he observed aggregates of red blood
cells in some combinations. He attributed this phenomenon to inherited individual differences and described the human blood groups A, B and O, followed by AB a year later (6). However, some patients still suffered from severe transfusion reactions during transfusion of compatible blood. Another important breakthrough was the discovery of the Rhesus (Rh) blood group system in 1940 (7). The existence of two distinct expression patterns of the RhD antigen on the surface of red blood cells (expression or not) also elucidated the etiology of hemolytic neonatal disease. The transfusion of ABO and RhD compatible blood to a patient reduced the incidence of dangerous hemolytic complications considerably. The transfusion of whole blood, which occurred until the 1960s, includes the transfer of donor white blood cells (leukocytes) to the patient. In the early 1950s, leukocyte antigens were discovered by agglutinating antibodies present in the sera of multitransfused and multiparous women suffering from febrile non-hemolytic transfusion reactions (8-11). It was soon found that these human leukocyte antigens (HLA) play an essential role in the human immune response and in transplant rejection. The correlation between the presence of leukocyte agglutinins and febrile transfusion reactions (12) was the basis for removal of leukocytes from blood transfusions for patients on dialysis or with blood diseases. In 1962 antibodies against HLA antigens were identified as a cause for platelet transfusion refractoriness. In the seventies blood component therapy was introduced that separated red blood cells (RBC), plasma and the buffy-coat by centrifugation. Removal of the buffy-coat, containing 50-80 percent of leukocytes and >90 percent of platelets, and the development of specialized leukocyte-reducing filters decreased the incidence of leukocyte-related complications considerably (13). However, in 1973 the importance of leukocytes in the immunomodulatory effect of pretransplant blood transfusions was recognized (14).

3. THE HUMAN IMMUNE SYSTEM

3.1 The immune response

Leukocytes are the principal cells of the immune system. They are able to ensure proper recognition and destruction of non-self molecules, such as bacteria and viruses, while responses to self molecules do not occur as these can cause autoimmune phenomena (1). In the absence of a functioning immune system, even minor infections can have a fatal outcome. However, this immune system is a barrier for blood transfusion therapy and transplantation. Foreign molecules (alloantigens) and cells present in the blood product or transplanted organ can activate the recipient’s immune system leading to transfusion complications or transplant rejection. The human immune system can be divided into the innate, non-antigen specific, immune system and the acquired, antigen-specific adapted, immune system.
3.1.1 Innate immunity
After passing the physical barriers of the skin and mucosal epithelia of the respiratory, gastrointestinal and reproductive tracts, the innate immune system provides an immediate, but non-specific response to invading pathogens. Proteins of the complement system that circulate in the blood or are locally produced will opsonize and kill particular pathogens. Cellular barriers of the innate immune system include natural killer (NK) cells, mast cells, basophils, eosinophils and phagocytes (macrophages, neutrophils and dendritic cells). NK cell activation is regulated by a balance between signals mediated through activating and inhibitory killer immunoglobulin-like (KIR) receptors (15). Inhibitory KIRs recognize the absence of self human leukocyte antigens (HLA) of the class I type (missing-self hypothesis) (16). In the absence of these ligands, such as in virus-infected host cells or transplanted donor cells (17), the balance shifts towards NK cell activation that may result in cytotoxicity. Phagocytes are able to engulf pathogens followed by the release of enzymes and acids that kill and digest the pathogen. Innate immune cells, like NK cells and dendritic cells, are also important mediators in the activation of the acquired immune system (18).

Blood transfusions interfere with the innate and acquired immune system. In stored blood products, granulocytes and macrophages deteriorate and become apoptotic or necrotic. Apoptotic cells, expressing annexin V/phosphatidylserine are engaged by macrophages that start to produce anti-inflammatory cytokines such as prostaglandin E2 and TGFβ. These factors suppress the proinflammatory innate immune response of macrophages and NK cells (19,20) and impair the function of dendritic cells, thereby influencing the acquired immune response as well (21).

3.1.2 Acquired immunity
The acquired immune system is antigen-specific and involves a cellular and humoral component. The cellular immune response is mediated by T lymphocytes. Via their T cell receptor (TCR), T cells specifically recognize the target antigen that is presented by professional antigen presenting cells (APCs), such as dendritic cells, B cells and macrophages. These APCs use molecules of the major histocompatibility complex (MHC), the human leukocyte antigens (HLA) in humans, for proper antigen presentation (22). T cells can activate other immune cells or become cytotoxic. Key players of the humoral immune response are B lymphocytes that carry an immunoglobulin receptor. Their principal function is the production of alloantibodies directed against soluble or cell surface antigens. Once T and B cells are triggered, a proportion of them will become memory cells. The memory cells will induce a fast and strong immune response upon a subsequent encounter with the antigen.

Blood transfusions contain many foreign antigens that can activate recipient T and B cells. Alloantigens can involve donor red blood cell molecules, foreign HLA molecules
or any other antigen present in the blood donor and not in the recipient. With increasing storage intervals of blood products, the quality of APCs deteriorates leading to a diminished ability to activate recipient T cells (21). Moreover, leukocyte-derived soluble factors accumulate in blood components upon storage. Pro-inflammatory cytokines, like interleukin (IL)-1, IL-6 and IL-8, activate recipient immune cells that may cause transfusion complications (23), whereas soluble HLA class I and II molecules and soluble Fas-ligand (FasL) may impair the function of T cells (24).

3.2 Human leukocyte antigens
Although the molecules of the HLA system were initially recognized as targets for immunological attack after blood transfusion and transplantation, the primary physiological role of HLA is to present peptides to T cells, thereby initiating the acquired immune response. The HLA system is the most polymorphic system described in humans (25,26). The high degree of polymorphism provides the human species with the best protection against the wide range of pathogens, as at least some individuals will carry the proper HLA molecule that can present pathogenic peptides to the immune system. The inheritance of two different sets of HLA molecules (one haplotype from the father and one from the mother) will further amplify this. Based on their structure and function in the immune response, HLA molecules are divided into two groups: class I and class II. HLA molecules are encoded by a cluster of genes located on the short arm of chromosome 6 (27).

3.2.1 HLA class I molecules
The classical HLA class I molecules (HLA-A, -B and -C) are composed of a heavy α-chain linked to a non-polymorphic light chain, β2 microglobulin, that stabilizes the complex (28). The α-chain consists of five domains: two peptide-binding domains (α1 and α2), one immunoglobulin-like domain (α3), the transmembrane region and the cytoplasmic tail (Figure 1A). The α1 and α2 domains are the most polymorphic and they form a peptide-binding groove for antigenic peptides of eight to ten amino acids in length that are primarily derived from endogenous proteins like self proteins or virus-induced proteins (29). All nucleated cells and platelets express HLA class I molecules and can be a target of an immune response. In circulating blood, about 70% of HLA class I antigens is expressed on platelets.

3.2.2 HLA class II molecules
The HLA class II molecules (HLA-DR, -DQ and -DP) consist of an α-chain and a β-chain, which form a heterodimer. Each chain consists of four domains: the peptide-binding domains (α1 and β1), an immunoglobulin-like domain (α2 and β2), the transmembrane region and the cytoplasmic tail (Figure 1B). The majority of the
polymorphism is located in the β1 domain of the HLA-DR molecules and in the α1 and β1 domains of the HLA-DQ and -DP molecules (30). These domains form a peptide-binding groove, which, in contrast to class I molecules, is open at both sides and can accommodate peptides of 13 to 25 amino acids in length (31). The peptides that bind to HLA class II molecules are mainly of exogenous origin. Before loading into HLA class II molecules as antigenic peptides, foreign proteins need internalization and processing by professional antigen presenting cells (APCs). HLA class II molecules are constitutively expressed on APCs, such as dendritic cells (DCs), macrophages and B cells, but also on activated T cells.

![Figure 1: HLA class I (A) and HLA class II (B) molecules.](image)

3.3 The activation of T cells
The HLA class I and class II molecules play a different role in T cell responses. The main function of HLA class I molecules is to present foreign peptides to CD8+ T cells that can mount a cytotoxic response (32). In contrast, the HLA class II-peptide complex is recognized by CD4+ T cells (33) that generally function as T helper cells. The structure of T cells that recognizes HLA molecules is the membrane-bound T cell receptor (TCR). The TCR complex of most T cells consists of an α and β chain that form a heterodimer and are linked to the non-polymorphic CD3 complex (34). A minority of T cells expresses a TCR composed of γ and δ chain. These γδ T cells possess some innate immune cell characteristics, such as the ability of recognizing microbial and lipid antigens without the need for presentation into HLA molecules (35). Besides triggering the TCR/CD3 complex (signal 1), T cells need co-stimulatory signals (signal 2) to become activated.
3.3.1 Direct versus indirect allorecognition

T cells can recognize HLA molecules via the direct or indirect pathway. Direct allorecognition (Figure 2A) refers to the recognition of intact allogeneic HLA class I and II molecules on the surface of donor APCs and is restricted to blood transfusion, transplantation and pregnancy. The strength of this type of immune response, as measured by precursor frequency of T cells, is about 100-fold higher than that of T cells recognizing alloantigens indirectly (36). Although the thymus only selects cells bearing self-HLA molecules, the structural similarity between the TCR contact surfaces of many HLA molecules may account for a situation in which an allogeneic HLA molecule with a peptide mimics self-HLA restriction (37).

Transfused donor APCs can give rise to direct allorecognition and trigger recipient T cells. Platelets express HLA class I molecules, but cannot activate T cells directly in the absence of donor APCs (38). Leukocyte-depletion and storage of the blood product can diminish the occurrence of direct allorecognition. In transplantation, the direct allorecognition pathway predominates in the first few weeks to months after transplantation and is the main cause of acute graft rejection (39,40). With elapsing time after transplantation, donor APCs fade away and the role for the indirect allorecognition pathway becomes more important.

Figure 2: Direct (A) and indirect (B) allorecognition pathway.

The indirect pathway of allorecognition reflects the normal mechanism of T cell stimulation by nominal antigens. Indirect allorecognition (Figure 2B) is the recognition of alloantigens in the context of self-HLA molecules present on self-APCs. Upon blood transfusion or transplantation, alloantigens are shed from donor cells or the graft, taken up by recipient’s APCs, degraded into peptides and presented in the groove of HLA
class II molecules to CD4+ recipient T cells. Alloantigens can be derived from any protein present in the blood or organ donor and not in the recipient, but mainly involves the HLA molecules and minor histocompatibility antigens (41). After blood transfusion or transplantation, self-APCs are permanently active to pick up donor-derived alloantigens and present these to recipient T cells. Evidence for the indirect pathway of allorecognition after transplantation came from observations that graft rejection still occurred in the absence of immunogenic donor-derived passenger cells in the graft (42). It is the dominant allorecognition pathway long after transplantation (43) and mainly associated with chronic graft rejection (44-47), which explains the need for life-long immunosuppressive drug use.

3.3.2 Co-stimulation
The interaction between co-stimulatory molecules on the membrane of the APC and the T cell leads to the development of an effective immune response. Two well-known co-stimulatory pathways are the CD28-B7 pathway and the CD40-CD40L pathway (48,49). Interaction between receptor and ligand will lead to full activation of the T cell. Upon activation, the T cell will synthesize and secrete interleukin (IL)-2, which drives clonal expansion and differentiation of the activated cell. To control the process of expansion of activated T cells, also inhibitory signals are necessary. Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) is homologous to CD28, but is an important negative regulator of T cell responses (50,51). Its expression is rapidly up regulated following T cell activation and results in termination of the T cell response.

3.4 Effector mechanisms

3.4.1 T cells
After engagement of the TCR and co-stimulatory signal, naïve CD4+ and CD8+ T cells can differentiate into effector cells, regulatory T cells and memory cells. The cytokines secreted by APCs determine the skewing of effector CD4+ T cells into T helper 1 (Th1) or Th2 cells. Generation of Th1 cells requires the presence of interferon γ (IFN-γ) and interleukin (IL)-12. These cytokines cause the Th1 cells to produce IFN-γ and tumor necrosis factor-β (TNF-β), thereby increasing the killing efficacy of macrophages and the proliferation of cytotoxic CD8+ T cells. Differentiation into Th2 cells will occur in the presence of IL-6 or IL-4, of which the latter can be released by NK cells. Th2 cells produce IL-4, IL-5 and IL-13 that will stimulate B cells to produce antibodies and will attract and activate eosinophils (52). Upon recognition of donor HLA class I antigens in presence of Th1 cytokines, CD8+ T cells acquire cytotoxic properties that enable them to kill their targets via the release of cytotoxic effector molecules, like perforin and granzymes, or via Fas-Fas ligand interaction (53).
3.4.2 B cells
Th cells are important initiators in the production of alloantibodies by B cells (54). B cells can bind and internalize donor antigens by their surface immunoglobulin receptor, cleave them into allopeptides and load the suitable ones into their own HLA class II molecules on the surface. However, these alloreactive B cells require a cognate interaction of Th cells to get activated. Upon antigen recognition by the T cell and engagement of CD40 on the B cell with CD40L on the T cell, the B cell will be able to divide and develop into an antibody producing plasma cell. Antibodies directed against donor HLA antigens are often responsible for complications after blood transfusion and rejection of the transplanted organ, via complement activation or antibody-dependent cell cytotoxicity (55).

3.5 Immune regulation
Regulation of normal immune responses involves central and peripheral mechanisms. Central tolerance results from thymic deletion of T cells that have the potential to respond to self antigens in a process called negative selection. As not all autoantigens may be present in the thymus and some cells may escape central deletion, multiple mechanisms contribute to tolerance in the periphery. Peripheral tolerance to autoreactive or alloreactive T cells can occur through clonal anergy, clonal deletion or active suppression or killing of alloreactive cells by regulatory cells (56).

T cells can become functionally inactive (anergic) when they are activated through the TCR in the absence of appropriate costimulatory signals (57) or through signaling via alternative receptors, like CTLA-4 (58,59). Clonal deletion can occur through persistent activation of the TCR that leads to activation-induced cell death (AICD). An important mechanism underlying the AICD in CD4+ T and B cells is the ligation of the Fas death receptor expressed on their surfaces by its ligand (FasL) on CTLs (60).

Active suppression of T cells can occur by particular populations of cells, including dendritic cells (DCs) and regulatory T cells. DCs, the most potent APCs, can also induce T cell tolerance, dependent on their maturation state. Antigen presentation by immature DCs is associated with induction of tolerance, due to the expression of low levels of HLA class II molecules and costimulatory molecules resulting in poor stimulation of T cells (61). Regulatory T cells (Tregs) can be derived from the thymus or arise as a result of tolerogenic factors present in the environment. The thymus-derived, naturally occurring regulatory CD4+ T cells express high levels of the IL-2 receptor α chain, CD25 (62). These CD4+CD25- T cells can suppress proliferation of other cells in a non-antigen-specific manner (63) and require direct cell-cell contact (64). The phenotypic markers CTLA-4 and forkhead box P3 (Foxp3) are associated with Tregs (65,66), but are not thought to be exclusive. The peripherally induced CD4+ Tregs, Tr1 and Th3, exert their suppressive function via IL-10 and TGFβ (67,68). CD8+ suppressor
T cells that lack CD28 expression (69), are able to induce the expression of inhibitory receptors, such as immunoglobulin like transcript 3 (ILT3) and ILT4, on APCs, rendering them tolerogenic and unable to stimulate CD4+ T cells (70).

4. CLINICAL CONSEQUENCES OF EXPOSURE TO FOREIGN HLA MOLECULES

4.1 Blood transfusion
The allogeneic character of blood transfusions can elicit harmful effector responses in the recipient. On the other hand, blood transfusions can lead to transfusion-related immunomodulation (TRIM) that encloses intended but also unintended effects (71).

4.1.1 Immune activation
Because erythrocyte transfusions are matched for ABO and RhD antigens, most adverse effects of blood transfusions can be attributed to differences in HLA. Adverse effects are primarily caused by HLA class I and class II antibodies produced by the recipient upon confrontation with donor leukocytes. Blood transfusion can also activate the alloreactive T cell compartment and increase the number of helper T cells and cytotoxic T cells (72,73), but the clinical sequelae are less clear.

The formation of HLA alloantibodies by the recipient (HLA immunization) depends on the blood product, recipient and donor (71). Characteristics of the blood product that favor immunization include the high number of viable leukocytes (74) and shorter storage intervals (21,24). Also the degree of HLA disparity determines immunization outcome in transfused patients. In case of two HLA class II mismatches, more frequent and broader HLA antibodies are developed, while sharing of an HLA haplotype or HLA-DR molecule results in less antibody formation and a lower CTLp frequency (75,76). Patients that have already developed alloantibodies due to previous confrontation with foreign HLA can boost such alloantibodies upon weaker stimulation. HLA alloantibodies form a major problem for patients that depend on platelet transfusions by causing immune destruction of transfused incompatible platelets, resulting in immune refractoriness to random donor platelet transfusions. Moreover, preformed HLA alloantibodies can destruct leukocytes in a subsequently transfused product and cause febrile non-haemolytic transfusion reactions (FNHTR).

Donor reactivity towards the recipient can result in two other severe transfusion complications. Transfusion-related acute lung injury (TRALI) is a complication resulting in pulmonary edema. It is caused by antibodies against HLA, granulocyte or monocyte antigens that can be found in the plasma of transfused blood components. Transfusion-associated graft-versus-host disease (TA-GVHD) is a lethal, but not common, complication and occurs in immunocompromised patients. Within five days
after transfusion, proliferating lymphoid blast cells of donor origin can be found in the recipient’s circulation (77). These cells can mount an immune response upon recognition of HLA or minor histocompatibility antigens expressed on host cells and often results in death of the patient (77). TA-GVHD can be prevented by gamma irradiation of cellular blood products that prevents proliferation of donor lymphocytes after stimulation by recipient cells (78).

### Table 1: Observed clinical effects after allogeneic blood transfusions.

<table>
<thead>
<tr>
<th>Immune activation</th>
<th>TRIM</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA immunization</td>
<td>Enhanced survival of transplanted organs</td>
</tr>
<tr>
<td>Febrile non-haemolytic</td>
<td>Cancer recurrence</td>
</tr>
<tr>
<td>transfusion reactions</td>
<td></td>
</tr>
<tr>
<td>(FNHTR)</td>
<td></td>
</tr>
<tr>
<td>Platelet transfusion</td>
<td>Increased incidence of postoperative infections</td>
</tr>
<tr>
<td>refractoriness</td>
<td></td>
</tr>
<tr>
<td>Transfusion-related</td>
<td>Increased short-term mortality in cardiac surgery</td>
</tr>
<tr>
<td>acute lung injury</td>
<td></td>
</tr>
<tr>
<td>(TRALI)</td>
<td></td>
</tr>
<tr>
<td>Transfusion-associated</td>
<td></td>
</tr>
<tr>
<td>graft-versus-host disease</td>
<td>(TA-GVHD)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Occurring in individual patients  
<sup>b</sup> Estimated from studies in patient cohorts

#### 4.1.2 Transfusion-related immunomodulation

Besides immune activation, allogeneic blood transfusions can suppress or modulate the recipient’s immune response, referred to as transfusion-related immunomodulation (TRIM) (79).

Immunosuppressive effects of allogeneic blood transfusions were reported over 30 years ago in patients who had undergone renal transplantation. This improved transplant outcome after allogeneic blood transfusions was frequently observed in the clinic but is not mechanistically clarified (see paragraph 5). If allogeneic blood transfusions were able to down-regulate the recipient’s immune system, it was feared that they might also decrease the mechanisms of cancer immune surveillance (80). In a meta-analysis, the hypothesis that perioperative allogeneic blood transfusions have a detrimental effect on recurrence of colorectal cancer was supported, but a causal relationship cannot be claimed due to wide differences in design and study population (81). Furthermore, blood transfusions during surgery come in a late phase of the disease, when immune surveillance already had its chance and failed. In addition, a large number of observational studies in humans found an increased incidence of postoperative bacterial infections after allogeneic blood transfusions. Again, a causal effect cannot be considered proven, but it cannot be excluded either (82,83). Until now, a deleterious effect of allogeneic leukocyte-containing blood transfusions is most consistently found in cardiac surgery and is associated with higher mortality due to
multiple organ failure (84). The clinical effects associated with allogeneic blood transfusions are summarized in Table 1.

4.1.3 Leukocyte-depletion
In order to obtain more pure blood products and reduce the occurrence of unintended effects, leukocyte-reducing strategies have been developed. From 1960 onwards, red blood cell and platelet blood transfusions have been (partially) leukocyte-depleted. From the late 1970s, additional methods and filters were developed that improved to decrease the number of leukocytes. The numbers of remaining leukocytes in red blood cell products are summarized in Table 2. Universal leukocyte-depletion (by filtration) of therapeutic blood products in the Netherlands was introduced in 2001 and based on the theoretical risk of transmitting variant Creutzfeldt-Jakob disease (85).

<table>
<thead>
<tr>
<th>Blood product</th>
<th>Leukocytes per unit (x10⁹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole blood (WB)</td>
<td>3</td>
</tr>
<tr>
<td>Plasma-reduced, red blood cell concentrate (RBC)</td>
<td>3</td>
</tr>
<tr>
<td>Buffy coat-depleted blood (BCD)</td>
<td>0.7-1.3</td>
</tr>
<tr>
<td>By-filtration-leukocyte-reduced blood (FLR)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

The depletion of leukocytes has led to several clinical benefits. It lowers the incidence of HLA immunization (74), platelet refractoriness (86) and FNHTR (87) considerably. However, approximately 15-20% of the recipients of leukocyte-depleted red blood cell or platelet transfusions still produce HLA alloantibodies (88). This can be due to the small number of remaining leukocytes in the blood product (38) or the indirect recognition of platelet-derived HLA class I alloantigens. In cardiac surgery, the incidence of mortality was significantly decreased in patients who received leukocyte-depleted blood products (84). On the contrary, leukocytes seemed to be required for the beneficial effect of allogeneic blood transfusions in transplant patients (14).

4.2 Transplantation
Upon transplantation the recipient is confronted with donor HLA class I molecules present on tissue cells and HLA class II molecules on passenger donor leukocytes in the graft. A major concern is the formation of HLA alloantibodies. Preformed HLA alloantibodies can cause graft rejection, but also HLA alloantibodies that develop during the lifetime of an allograft. By performing a cross-match before transplantation with donor cells and the patients’ serum, antibodies can be detected. A positive cross-match, due to complement fixing HLA antibodies of the IgG class, is considered a
contraindication for transplantation (89). Graft rejection can be divided into three types: hyperacute, acute and chronic rejection.

4.2.1 Transplant rejection

Hyperacute rejection occurs within minutes or hours after transplantation and is mediated by existing antibodies. ABO and/or HLA alloantibodies can bind to endothelial cells on the graft and activate the complement pathway (90). Acute rejection is primarily a T cell mediated process and occurs within the first 3 months after transplantation. T cells are the main cellular infiltrate seen in the biopsies of acute rejected grafts and the current immunosuppression protocols directly target these T cells. Recently it was found that biopsies taking during acute rejection episodes also showed positive staining for the complement product C4d, which suggests a role for antibodies in acute rejection as well (91). In kidney transplantation, chronic rejection is currently the most prevalent cause of transplant failure (92). It develops months or years following transplantation and is characterized by a slow deterioration of graft function. The exact pathogenesis of chronic rejection is incompletely understood and thus treatment is difficult. It is presumed that indirect allorecognition is the predominant immunological driving force able to induce chronic cellular and humoral effector mechanisms (93,94).

4.2.2 HLA matching and immunosuppressive drugs

Matching for HLA molecules between donor and recipient improves kidney and heart allograft survival, with the strongest beneficial effect for matching the HLA-DR molecules (95-98). However, due to the enormous polymorphism of HLA, matching is in most cases not possible (25,26).

Immunosuppressive drug therapy is necessary in genetically non-identical transplant patients. Immunosuppressive agents act by inhibition of various steps of the T cell activation pathway and are used as induction, maintenance or rejection therapy. Induction therapy is an intense, prophylactic therapy given at the time of transplantation to reduce acute rejection in the first days to weeks after transplantation (99,100). Maintenance therapy usually consists of a combination of agents and has to be used lifelong. Nowadays, immunosuppressive protocols tend to minimize or withdraw the use of these agents as they all act nonspecifically (101). They are associated with serious side effects, such as an increased risk of infections and certain malignancies (102,103). Therefore, the ultimate goal is to achieve and maintain specific transplantation tolerance without the need for nonspecific immunosuppressive drugs.
5. IMMUNOMODULATION BY PRETRANSPLANT BLOOD TRANSFUSIONS

5.1 The blood transfusion effect

Allogeneic blood transfusions have been associated with transplantation tolerance, since the observation in 1973 by Opelz and colleagues of improved kidney graft survival in patients who received multiple transfusions from random donors (3). Better graft survival was associated with an increased number of transfusions in a dose-dependent manner (104). It was presumed that the appearance of HLA alloantibodies in multi-transfused patients accounted for the improved graft survival by selection of a cross-match negative graft. However, in 1979 it turned out that a single blood transfusion was also able to improve graft survival, but requires the presence of leukocytes (14). After this observation, research focused on the need for HLA compatibility between blood donor and recipient. Patients transfused with, fresh or frozen, (partly) HLA-DR shared blood showed an enhanced kidney or heart allograft survival compared with patients that received a HLA-DR mismatched transfusion (75,105). Moreover, the incidence of HLA immunization and CTL formation was lower in recipients of an HLA-DR matched transfusion as compared with an HLA-DR mismatched transfusion (106,107). An additional requirement for the beneficial effect seems to be HLA class II disparity on the other haplotype (108).

An overview of studies that investigated the effect of pretransplant blood transfusions on the function and survival of organ transplants from deceased donors is given in Table 3. Although the beneficial transfusion effect seems to be present despite the use of modern immunosuppressive drugs, deliberate pretransplant transfusions are currently virtually abandoned. The main reasons are concern for the development of HLA alloantibodies that hamper transplantation in the living-related setting and the transmission of infectious diseases.

In living-related kidney transplantation, pretransplant blood transfusions are applied with blood from the prospective organ donor. This protocol was initiated in an attempt to select potentially successful transplants by measuring the HLA sensitization rate after donor-specific blood transfusions (DST). It turned out that these DST improved graft outcome as well (126,127). However, a major risk factor is the development of HLA alloantibodies upon transfusion that hamper transplantation with that specific donor. To prevent this HLA immunization, most DST were administered under the coverage of various types of immunosuppressive drugs. There are two possible mechanisms of the success of the donor-specific transfusion protocol. A first explanation may be the process of selection, while this protocol separates responders from non-responders by means of monitoring the specific antibody response after transfusion. Another explanation may include the immunomodulatory effect(s) of DST.
Table 3a: Effect of pretransplant blood transfusions on clinical outcome after kidney transplantation.

| Reference          | Blood transfusion | No (n) | Yes (n) | Blood product (type, number and HLA compatibility) | Study  | Clinical outcome after kidney transplantation  
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Acute rejection (%)</td>
</tr>
<tr>
<td>Opelz et al. (3)</td>
<td></td>
<td>25</td>
<td>57</td>
<td>BCD &gt;10/S random</td>
<td>Retro</td>
<td>n.a.</td>
</tr>
<tr>
<td>Persijn et al. (14)</td>
<td></td>
<td>74</td>
<td>30</td>
<td>n.a. 1/S random</td>
<td>Retro</td>
<td>n.a.</td>
</tr>
<tr>
<td>Persijn et al. (14)</td>
<td></td>
<td>19</td>
<td>8</td>
<td>BCD$^1$ 1/F random</td>
<td>Pros</td>
<td>n.a.</td>
</tr>
<tr>
<td>Sanfilippo et al. (109)</td>
<td></td>
<td>22</td>
<td>8</td>
<td>RBC$^2$ &gt;1/S random</td>
<td>Pros</td>
<td>n.a.</td>
</tr>
<tr>
<td>Lundgren et al. (110)</td>
<td></td>
<td>19</td>
<td>8</td>
<td>BCD &gt;1/S random</td>
<td>Pros</td>
<td>n.a.</td>
</tr>
<tr>
<td>Melzer et al. (111)</td>
<td></td>
<td>49</td>
<td>163</td>
<td>WB/RBC &gt;1/S random</td>
<td>Retro</td>
<td>n.a.</td>
</tr>
<tr>
<td>Kerman et al. (112)</td>
<td></td>
<td>220</td>
<td>334</td>
<td>RBC &gt;1 random</td>
<td>Retro</td>
<td>n.a.</td>
</tr>
<tr>
<td>Taga et al. (75)</td>
<td></td>
<td>41</td>
<td>32</td>
<td>WB/BCD 1/S 1 DR match$^1$ random</td>
<td>Retro</td>
<td>n.a.</td>
</tr>
<tr>
<td>Middleton et al. (114)</td>
<td></td>
<td>29</td>
<td>30</td>
<td>RBC$^2$ 1 DR match$^1$ random</td>
<td>Pros</td>
<td>n.a.</td>
</tr>
<tr>
<td>Bayle et al. (115)</td>
<td></td>
<td>83</td>
<td>119</td>
<td>WB$^1$ 1/S haplo-identical$^1$ random</td>
<td>Retro</td>
<td>11% vs 32%$^c$ p=0.01</td>
</tr>
<tr>
<td>Opelz et al. (16)</td>
<td></td>
<td>218</td>
<td>205</td>
<td>RBC 3/S random</td>
<td>Pros</td>
<td>55% vs 54% n.s.$^c$</td>
</tr>
<tr>
<td>Christiaans et al. (117)</td>
<td></td>
<td>44</td>
<td>59</td>
<td>BCD$^1$ 1/F DR match$^1$ random</td>
<td>Retro</td>
<td>27% vs 27% n.s.$^c$</td>
</tr>
<tr>
<td>Mariat et al. (118)</td>
<td></td>
<td>49</td>
<td>107</td>
<td>RBC$^1$ 1/F DR match$^1$ random</td>
<td>Pros</td>
<td>65% vs 72% n.s.$^c$</td>
</tr>
<tr>
<td>Hiesse et al. (119)</td>
<td></td>
<td>36</td>
<td>31</td>
<td>BCD$^2$ 1/F DR match$^1$ random</td>
<td>Pros</td>
<td>33% vs 19% n.s.$^c$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>39</td>
<td>39</td>
<td>BCD$^2$ 1/F no DR match$^1$ random</td>
<td>Pros</td>
<td>33% vs 33% n.s.$^c$</td>
</tr>
</tbody>
</table>

WB=Whole Blood. RBC=Red Blood cell Concentrate. BCD=Buffy Coat-depleted. FLR=by-Filtration-Leukocyte-Reduced blood. F=fresh (<72h). S=stored. Retro=retrospective. Pros=prospective. $^*$ randomized controlled trial. n.a.=no information available. n.s.=not significant (p>0.05). $^a$ no blood transfusion vs blood transfusion, unless noted. $^b$ severe, recurrent rejection episodes. $^c$ number of rejections/number of transplantations. $^d$ 1-year graft survival. $^e$ 2-year graft survival. $^f$ 3-year graft survival. $^g$ 5-year graft survival.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Blood transfusion</th>
<th>Blood product (type, number and HLA compatibility)</th>
<th>Study</th>
<th>Clinical outcome after organ transplantation$^{a}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No (n)</td>
<td>Yes (n)</td>
<td></td>
<td>Acute rejection (%)</td>
</tr>
<tr>
<td><strong>HEART</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Keogh et al. (120)</td>
<td>21 9 n.a.</td>
<td>&gt;1 random</td>
<td>Retro</td>
<td>0.19 vs 0.09$^{c}$</td>
</tr>
<tr>
<td>Katz et al. (121)</td>
<td>29 39 RBC</td>
<td>&gt;1 random</td>
<td>Retro</td>
<td>n.a.</td>
</tr>
<tr>
<td>Kerman et al. (122)</td>
<td>65 72 n.a.</td>
<td>1-4 random</td>
<td>Retro</td>
<td>n.a.</td>
</tr>
<tr>
<td>Lagaaij et al. (75)</td>
<td>10 BCD$^1$</td>
<td>1 DR match$^1$ no DR match$^2$</td>
<td>Pros</td>
<td>30% vs 90%$^{2}$</td>
</tr>
<tr>
<td>Van der Mast et al. (105)</td>
<td>45 55 BCD$^1$</td>
<td>1/F 1 DR match$^1$ no DR match$^2$</td>
<td>Retro</td>
<td>31% vs 53%$^{2}$</td>
</tr>
<tr>
<td><strong>PANCREAS-KIDNEY</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stratta et al. (123)</td>
<td>45 61 n.a.</td>
<td>&gt;1 random</td>
<td>Retro</td>
<td>64% vs 47%</td>
</tr>
<tr>
<td><strong>LIVER</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Koneru et al. (124)</td>
<td>25 96 RBC</td>
<td>&gt;1 random</td>
<td>Retro</td>
<td>52% vs 50%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>40% vs 22%</td>
</tr>
<tr>
<td>Parker et al. (125)</td>
<td>30 30 RBC$^1$</td>
<td>&gt;4 random</td>
<td>Retro</td>
<td>67% vs 40%</td>
</tr>
</tbody>
</table>

RBC=Red Blood cell Concentrate; BCD=Buffy Coat-depleted; FLR=Filteration-Leukocyte-Reduced Blood; F=fresh (<72h); S=stored; Retro=retrospective; Pros=prospective. n.a.=no information available; n.s.=not significant (p>0.05). $^{a}$no blood transfusion vs blood transfusion, unless noted. $^{b}$severe, recurrent rejection episodes. $^{c}$number of rejections/number of transplantations. $^{d}$kidney graft survival. $^{e}$pancreas graft survival. $^{f}$Fisher’s exact test; original article Wilcoxon rank sum test: p<0.05
5.2 Immunomodulatory mechanisms

Although possible mechanisms of immunomodulation have been extensively studied, it is still not known how blood transfusions can down-regulate the alloimmune response upon transplantation.

The mechanisms that have been proposed are based on the suppression of alloreactive recipient innate and acquired immune cells that may be harmful for the subsequent organ transplant (Table 4) (128,129).

<table>
<thead>
<tr>
<th>Proposed mechanisms of the blood transfusion effect.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clonal deletion of alloreactive cells (apoptosis)</td>
</tr>
<tr>
<td>Anergy induction of alloreactive immune cells</td>
</tr>
<tr>
<td>Polarization of the immune system towards a Th2 response</td>
</tr>
<tr>
<td>Induction of suppressor cells</td>
</tr>
<tr>
<td>Production of anti-idiotypic antibodies</td>
</tr>
<tr>
<td>Microchimerism</td>
</tr>
</tbody>
</table>

Basically, these mechanisms are based on the elimination or inactivation of recipient immune cells. Elimination of potentially reactive recipient T cells can occur when soluble molecules, such as soluble FasL (sFasL) and soluble HLA molecules, are released by stored donor cells in the blood product (24).

sFasL can induce apoptosis of recipient cells by binding to Fas molecules expressed on recipient NK cells and cytotoxic T cells (130). Soluble HLA molecules can enter the recipient’s thymic circulation and cause clonal deletion of alloreactive recipient T cells (131).

Inactivation of recipient immune cells can occur when they become unresponsive (tolerant) for activation signals or when other cells suppress their action. It is reported that storage of blood products favors tolerance induction, while donor leukocytes change and APC lose their ability to deliver proper co-stimulatory signals upon storage (21). This can lead to the induction of a state of anergy, which is characterized by unresponsiveness of alloreactive recipient T cells. Moreover, stimulation by allogeneic blood transfusions can skew the recipient immune system towards a Th2 phenotype.

Cytokines produced by Th2 cells (IL-4, IL-5 and IL-13) and TGFβ are capable of down-regulating Th1 activities, such as antigen processing, macrophage activation and cytotoxic T cell activation (132).

The sharing of HLA-DR molecules between blood donor and recipient can also promote tolerance. In an experimental setting, more IL-10 secretion and efficient IFNγ and TNFα inhibition was found after activation of responder cells with an one-HLA-DR matched donor as compared with a complete mismatched donor (133). Additionally, it is suggested that donor APCs that share an HLA-DR molecule with the
recipient induce regulatory T cells that may suppress an immune response towards the subsequently transplanted organ (see also chapter 3) (134,135). It is thought that suppressor T cells, or regulatory T cells, play an important role in transplantation tolerance (136). Also anti-idiotypic antibodies and blocking antibodies after transfusion are described that can fulfill a role in tolerance induction (137,138).

A feature of tolerance may be the persistence of a low percentage of donor cells in the recipient after transfusion (microchimerism) (139-141). It is not exactly known if it is a cause or effect of immunologic tolerance, but it is thought that a tolerant state must exist in order for donor cells to persist. In patients that have a profound suppression of the innate immune system due to trauma or surgery (142), donor leukocytes can persist for many years, even after leukocyte-depleted blood transfusions (143). Partially (HLA-DR) shared allogeneic cells favor microchimerism and can be detected in the recipient's circulation up to eight weeks after transfusion (144).

6. AIM OF THIS THESIS

The initial reports on the beneficial effects of allogeneic blood transfusions upon organ transplantation in humans go back to the early 1970s. From that time, several studies attempted to confirm these effects and speculate about the mechanisms. Since the generation of new immunosuppressive drugs questioned the additional benefits of pretransplant allogeneic blood transfusions, many centers discontinued the administration of blood before transplantation. Our center continued this procedure for patients on the waiting list for simultaneous pancreas-kidney transplantation (SPKT). In Chapter 2 we showed that in this patient population an HLA-DR shared blood transfusion was able to diminish the severity of acute rejection episodes after SPKT. The need for HLA-DR sharing, as shown in the literature and in our study, suggests a role for the indirect allorecognition. Our hypothesis focuses on the role of indirect allorecognition after transfusion in improved transplantation outcome and is discussed in Chapter 3. To be able to investigate T cells with indirect allospecificity before and after a pretransplant protocolled blood transfusion, we aimed to develop an in vitro model for the indirect allorecognition. Our results, together with the possibilities and pitfalls of current approaches to measure indirect recognition of alloantigens are discussed in Chapter 4. Blood transfusions are able to activate as well as modulate the recipient’s immune system, but the requirements for each direction are not known. To determine the different effects of blood transfusions, we obtained blood from patients before and after a transfusion with fresh, leukocyte-rich blood from a donor matched for one HLA-DR antigen. Additionally, blood was obtained from patients that received another type of pretransplant transfusion, i.e. a donor-specific transfusion (DST), in the living-related kidney transplantation setting.
Chapter 1

Results of stimulation of patient cells with donor and third party as measured by current read out systems are described in Chapter 5. In order to be able to determine the incidence of chimerism after an HLA-DR shared blood transfusion containing leukocytes, different techniques were compared to monitor the presence of donor cells after transfusion (Chapter 6). Finally, results of this thesis are summarized and discussed in Chapter 7.
References


51. Walunas TL, Lenschow DJ, Bakker CY et al. CTLA-4 can function as a negative regulator of T cell activation. Immunity 1994;1: 405-413.


Chapter 1


