SUMMARY AND GENERAL DISCUSSION
SUMMARY

The question addressed in this thesis was: does a beneficial effect of pretransplant blood transfusions still exists and if so, are we able to unravel the immunological mechanism involved with modern in vitro techniques? It is known that blood transfusions can lead to immunization or tolerance in the recipient. The latter is characterized by an improved transplant outcome after pretransplant blood transfusions and was the basis of our research. The first observations of improved kidney graft function and survival after blood transfusion date 35 years back (1) and were later reported in heart, liver and pancreas-kidney transplantation as well. Despite intensive research, no exclusive immunological mechanism has been found. Moreover, investigation was hampered by abolition of pretransplant transfusion policies in many hospitals that observed an improved transplant outcome due to better rejection diagnosis, patient care and immunosuppressive drugs. The fact that late side effects of immunosuppressive drugs and chronic graft rejection remain a major problem emphasizes the importance of developing tolerance-inducing strategies. If we can identify factors that promote transplantation tolerance, graft acceptance may improve and immunosuppressive drugs can be withdrawn or reduced. In this respect, understanding of the immunological mechanism of the blood transfusion effect would be helpful to develop tolerance-inducing strategies. That was the final aim of our clinical and fundamental research on the effects of pretransplant blood transfusions.

A summary of clinical studies investigating the effect of pretransplant blood transfusions on the occurrence of acute rejection episodes and graft survival after kidney, heart, pancreas-kidney and liver transplantation is given in Chapter 1 (Table 3). The majority of the studies found a beneficial transfusion effect, but the heterogeneous character of the studies should be noted. At the Leiden University Medical Center (LUMC), a pretransplant protocolled blood transfusion (PBT) protocol still exists for non-immunized patients on the waiting list for simultaneous pancreas-kidney transplantation (SPKT). We investigated the effect of this transfusion protocol on the occurrence of acute kidney graft rejections and patient and graft survival (Chapter 2). The major intervention that was responsible for decreasing the percentage of patients with acute rejection episodes of the kidney graft was treatment with induction therapy. Development of subsequent graft rejections, defined as more severe rejections that require ATG treatment, was prevented by PBTs in 50% of the patients irrespective of the use of induction therapy.

Patients in the LUMC were transfused with a pretransplant blood product that contained leukocytes and was matched for one HLA-DR antigen with the recipient. Many immunological mechanisms are proposed for the beneficial transfusion effect,
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but most of these cannot explain the difference between HLA-DR matched and mismatched transfusions. In Chapter 3, an immune mechanism is described, which may explain the central role for shared HLA-DR molecules in the beneficial transfusion effect.

HLA-DR molecules on antigen presenting cells (APCs) of the transfusion donor can be recognized by recipient T cells via the direct (allo HLA) or indirect pathway (self HLA + allopeptide). The observation that HLA-DR sharing between blood donor and recipient favors a beneficial transplant outcome, suggests that the indirect way of allore cognition is important. While generally accepted assays exist to measure recipient T cells with direct allospecificity, a reliable and robust assay for monitoring T cells with indirect allospecificity is lacking, although many assays have been described for this purpose. With in vitro assays using T cell clones with indirect allospecificity and different sources of donor alloantigen, we have analyzed the advantages and pitfalls of the published approaches and concluded that a robust assay is still lacking (Chapter 4). In an attempt to unravel the immunological mechanism of the blood transfusion effect, phenotypic and functional in vitro assays were performed with recipient peripheral blood mononuclear cells (PBMCs) before and after an HLA-DR shared PBT (Chapter 5). After stimulation with blood donor PBMCs in an Elispot, we detected an increase of recipient memory IFNγ producing T cells 2 weeks (2wk) after blood transfusion as compared to pre-transfusion. This immune activation by PBT was not accompanied by changes in phenotypic cell surface or intracellular markers. Additionally, similar analyses were performed, but in another pretransplant transfusion setting. Patients waiting for living-related kidney transplantation received a donor-specific transfusion (DST) in case they had previously been immunized by pregnancy. Again, an increased IFNγ production was observed 2wk after DST. In contrast to PBT, DST was more potent in activating the recipient’s immune system, as increased frequencies of IFNγ producing cells were also found longer after DST and after third party stimulation. Phenotypic analysis showed that 2wk post-DST the number of NK cells increased significantly, which was accompanied by an increased expression of NK cell-related genes in microarray analysis. Moreover, microarray analysis showed many more activated immune genes in DST recipients, but not in PBT recipients. The distinct immunological profile of PBT and DST recipients (non-immunized versus immunized by pregnancy, respectively) may account for the different in vitro observations.

A feature associated with immunological tolerance after transplantation is microchimerism (2). The persistence of a low percentage of donor cells in the recipient can lead to transplantation tolerance to other tissues or organ from the same donor, as established in rodents, large animal models and humans. Microchimerism after blood transfusion may be one of the mechanisms of the beneficial blood transfusion effect after transplantation. In Chapter 6 we detected microchimeric cells of blood donor
origin in the peripheral blood of a patient 2wk after PBT by use of the nested PCR technique. The nested PCR is a highly sensitive assay, but associated with the risk of false-positive reactions. Therefore, we developed a new approach that combines flowcytometric cell sorting and quantitative PCR. It is based on sorting the chimeric cell population by using HLA-specific monoclonal antibodies for subsequent quantitative analysis in real-time PCR to confirm donor origin. The principle of this technique is described in Chapter 6. Taken together, this thesis includes the first observations of a beneficial effect of pretransplant blood transfusions in simultaneous pancreas-kidney transplantation. However, despite current available sophisticated in vitro techniques we were not able to explain the immunological mechanism involved. In particular, no suggestion for regulatory T cell establishment could be found. Our investigations into the indirect allorecognition and microchimerism may contribute to the development of reliable assays that can be used in the field of transplantation tolerance.

GENERAL DISCUSSION

Clinical evidence for a beneficial blood transfusion effect
An overview of clinical studies investigating the effect of pretransplant blood transfusions on the function and survival of transplanted organs from deceased donors is given in Table 3 of Chapter 1. Of the 13 clinical studies that compared patients who received a pretransplant blood transfusion with non-transfused patients, eight observed less acute rejection episodes and/or improved graft survival after transfusion (1,3-9). Within the time-frame of these studies, graft outcome improved as well due to better medical facilities, but could still be affected by pretransplant blood transfusions (6). Taken into account the presence of leukocytes or sharing of an haplotype or HLA-DR antigen between blood donor and recipient, possibly promoting hyporesponsiveness upon transplantation (10), eight of the eleven studies showed better clinical outcome (3,5,11-15). In SPKT, only two retrospective studies have been performed, including ours. We found that patients benefit from one unit of HLA-DR shared blood before SPKT, as they developed fewer severe acute rejection episodes needing treatment with ATG than patients without a pretransplant transfusion (16). In the other study only a trend, but not a significantly lower rejection rate after random transfusions was found, without improvement of one year graft survival (17).

In living-related kidney transplantation DSTs have a beneficial effect as well (18). As the organ donor is not always selected on basis of HLA compatibility, the blood product not necessarily shares an HLA antigen with the recipient. However, blood and organ donor are from the same individual, which is thought to promote a beneficial outcome (19). Marti et al. depicted the clinical benefit from DST as observed in many studies, but also highlighted the heterogeneous character of the studies (18).
The huge heterogeneity between the studies (investigating the effect of PBT as well as DST) is caused by the time frame of more than 30 years during which many clinical improvements were made. Due to the retrospective character of almost all studies, they differ in selection of patients, blood product, presence or absence of various types of immunosuppressive coverage and duration of follow-up. While outcome after transplantation depends on several factors that cannot be controlled in a retrospective study, it is difficult to attribute a favorable effect to a pretransplant blood transfusion, especially in studies that did not include a control group or based their conclusions on historical control groups. Also in our retrospective study a good control group (without pregnancy or therapeutic transfusions) is lacking.

Although well-designed observational studies with a retrospective character are valuable in clinical research to generate a hypothesis, randomized controlled trials (RCTs) are needed to confirm the assumptions (20). Only three RCTs investigated the pretransplant blood transfusion effect in deceased-donor organ transplant recipients (see Table 3). Two smaller studies (≤40 patients per group) did not observe a better transplant outcome after a random or partly HLA-DR matched transfusion respectively (21,22). However, the first one did not take into account acute graft rejection, which is a risk factor for chronic rejection and may be missed when looking at 1-year graft survival, while the second one may be underpowered to detect an effect of HLA-DR shared blood transfusions on graft outcome. The only RCT that observed a clinical benefit of pretransplant blood transfusions was a large multi-center study (≥200 patients per group), where patients experienced less often severe acute rejection episodes and a higher 5-year graft survival after having received three randomly selected, stored blood products (6). The difference in the outcome parameters between transfused and non-transfused patients was however small, less than 10%. In living-related kidney transplantation, only one small study with a prospective randomized design has been performed (23). In that study, 15 patients received a DST 24h pre-operatively with cyclosporin A coverage and the remaining 15 subjects did not receive a DST. The DST group demonstrated significantly fewer acute rejection episodes, a markedly better graft function and a clear trend toward a higher 1-year allograft survival.

Although many observational clinical studies observed a possible immunosuppressive effect of pretransplant blood transfusions, its effect has not been proven in sufficiently powered RCTs.

Determination of an effect of a DST or PBT on transplant outcome requires RCTs considering the following factors:

- Selection of a well-designed patient cohort and control group.
Summary and general discussion

- Power. While transplant outcome is influenced by many factors and a pretransplant blood transfusion may be one of these, the study should include enough patients to demonstrate a transfusion effect in a multivariate analysis. For SPKT in the Netherlands this is impossible, as there are less than 30 transplantations per year.

- Standardization of the blood product. It is assumed that leukocytes are required (3), but in this respect different blood products have been used (whole blood, red blood cell concentrates, Buffy coat-depleted blood). Additionally, the exact number of leukocytes/kg bodyweight recipient in each blood product should be standardized.

- Characteristics of the blood product. This depends on the research question. Fresh as well as stored blood products have been shown to improve graft outcome, but this may be due to different mechanisms. Only one RCT focused on the transfusion of a distinct one HLA-DR shared blood product (22), whereas many assumptions exist about the relevance of sharing one HLA-DR antigen with the recipient. A RCT that have not been performed yet is a RCT that involves one arm with patients who received HLA-DR matched blood, one arm with patients who received HLA-DR mismatched blood and one arm with non-transfused patients.

- Transfusion protocol. The number of administered products differ in many studies, but it has been observed that one unit containing viable leukocytes may already be of clinical benefit (3). Also, the time-interval between transfusion and transplantation has been questioned before. Blood transfusions given immediately before or during the transplantation of organs from deceased donors were less effective than blood transfusions given 2wk before transplantation (24), whereas one RCT established the opposite effect in recipients of a DST given 24h before living-related kidney transplantation (21). An influence on the time-interval in case of organ transplantation of deceased donors can however not be managed.

- Immunosuppressive drug therapy. The development of immunosuppressive drugs is a continuous process. However, immunosuppressive drug treatment should be standardized within one study as it influences transplant outcome to a great extent.

- Follow-up. Most studies investigated the effect of a PBT or DST on acute graft rejection and on one-year graft survival. More information can be obtained when the occurrence of severe acute graft rejections, chronic rejection and long-term graft survival are also analyzed.

Towards the mechanism

The variety in study characteristics also hampers understanding the possible mechanisms of the beneficial effect of pretransplant blood transfusions. Different mechanisms have been described explaining the observations that random blood
transfusions compared to none and HLA-DR matched transfusions compared to HLA-DR mismatched transfusions favor transplantation tolerance. Elimination or suppression of alloreactive recipient immune cells can occur after the transfusion of stored blood products (25). Soluble mediators released upon storage, e.g. soluble Fas ligand or soluble HLA molecules, or the lower capacity of antigen presentation after storage of donor APCs can turn recipient T cells into anergic cells or cells that undergo clonal deletion (25,26). However, as several studies found a beneficial effect with fresh (<72h stored) blood products, down-regulation of costimulatory signals, occurring after two weeks of storage time, may only be part of the mechanism. It can also not explain the differences in transplant outcome following HLA-DR compatible versus incompatible blood transfusions.

Pretransplant blood transfusions can have an activating effect (HLA immunization) or suppressive effect (27). Our in vitro experiments showed that two weeks after transfusion IFNγ producing T cells can be found, which is in line with other studies (28-31). The question is how these alloreactive recipient T cells would lead to hyporesponsiveness towards a subsequent organ donor. Terasaki hypothesized that the highly reactive T cell clones that are generated upon a secondary stimulus by transplantation are deleted by high-dose immunosuppression (32). The observation that some HLA disparity between recipient and transfusion donor is necessary for the beneficial transfusion effect fits in this hypothesis (19,33). However, the hypothesis cannot explain an improved transplant outcome after HLA-DR matched (for one antigen) versus mismatched transfusions. Besides immunosuppressive drugs, alloreactive T cells may be suppressed by biological factors, such as regulatory T cells (Tregs). In DST treated animals, active suppression by Tregs seemed to play a role, as an early immune activation period after transplantation was followed by hyporesponsiveness in the later post-transplant period (34). Moreover, it was found that tolerated grafts contain dense cellular infiltrates (35) that are even able to kill donor target cells (36). It has been shown that Tregs, induced by DST which can transfer donor-specific tolerance to naïve animals, require interleukin (IL)-10 for functional activity (37) and can regulate in an antigen nonspecific manner (38). Tregs have been generated after DST (39), but also following multiple transfusions of blood isolated from donor strains unrelated to the eventual allograft donor (40).

The question arises why exposure to allogeneic cells by blood transfusions would generate Tregs at all. First, T cell activation may include the Treg compartment, influenced by donor cell persistence for sufficient time after blood transfusion in order for regulation to develop. The increased percentage of surviving grafts after multiple blood transfusions compared with single ones, suggests that after a single blood
transfusion donor cells may be cleared before Tregs could be generated (40). However, it cannot be excluded that the higher rate of HLA immunization after multiple blood transfusions had led to the selection of better matched grafts leading to improved survival. Second, the delivery of blood donor-derived alloantigens intravenously may engage naturally occurring mechanisms that contribute to normal peripheral tolerance (40). If alloantigen recognition takes place in the absence of inflammation or other danger signals (41), it may become part of a peripheral tolerance process. Third, it is hypothesized that the sharing of HLA-DR antigens between recipient and blood donor favors Treg induction (42,43). The shared HLA-DR antigens on donor APCs activate recipient CD4+ T cells when they contain a foreign peptide as if presentation on self HLA had occurred (indirect alloantigen recognition). Such activated CD4+ T cells possess the capability of suppressing other alloreactive cells. CD4+ T cells specific for a self-HLA-DR antigen containing a foreign peptide were capable of down-regulating autologous cytotoxic T cells via cell lysis or production of the inhibitory cytokine IL-10 (44). This is in line with the observation that higher IL-10 secretion was observed in a mixed lymphocyte culture in case of stimulation with 1 HLA-DR matched cells, in contrast to complete HLA-DR disparity (45). A beneficial transplant outcome in humans after an one HLA-DR shared blood transfusion may thus rely on the induction of Tregs together with a diminished risk of inducing HLA alloantibodies (46) and cytotoxic T lymphocytes (46,47). Similar immunological changes were also observed when tolerance was established in the prenatal period (neonatal tolerance). Exposure to the non-inherited maternal HLA antigens (NIMAs) on maternal cells during pregnancy or breast feeding was associated with a reduced HLA antibody formation against the NIMA (48) and a better survival of grafts from donors that were mismatched for the NIMA haplotype as compared to the non-inherited paternal HLA antigens (NIPA) (49-51). Also in case of fetal-maternal tolerance, Tregs are thought to play an important role (52). Recently, it was found that CD4+CD25hiFoxp3+ Tregs could be generated in utero against NIMA, leading to a suppressed fetal anti-maternal immune response (53). In some cases, these Tregs even persisted until early childhood.

Although there is evidence that CD4+ T cells with indirect allospecificity can regulate other cells, it is still not demonstrated that these CD4+ Tregs are induced in humans by a pretransplant blood transfusion. To identify such CD4 populations, we aimed to measure T cells with indirect allospecificity in vitro. As extensively discussed in Chapter 3, this failed due to lack of reliable tests. Our next approach to detect Tregs after pretransplant blood transfusion focused on phenotypic analysis of pre- and post-transfusion samples. The percentage of Tregs (phenotypically defined as CD4+CD25hiFoxp3+ cells) remained the same after transfusion in all DST and PBT recipients, also after restimulation in vitro with cells from the transfusion donor.
We were not able to provide evidence for the induction of Tregs by pretransplant blood transfusions. Still, Tregs may play an important functional role, despite this was not reflected in an increase in number in the peripheral blood.

For Tregs to exert their regulatory function, they have to be in close contact with donor-reactive cells (54). Important sites of action are therefore secondary lymphoid organs and the transplanted organ itself. It was described that well-functioning Tregs can be found in the peripheral blood, but they may disappear from the periphery to other compartments when their suppressive effect is needed locally (55). Animal studies showed that donor-specific Tregs induced by DST accumulate in tolerated grafts (56) and need a second trigger by the graft for sufficient numbers to develop and protect from rejection (57). In humans, a recent study found significantly more Foxp3+ T cells in biopsies of renal allografts after DST than without DST, an observation especially salient in biopsies with signs of acute rejection (58). A role for DSTs in the recruitment of Foxp3+ cells was suggested, but a direct influence of Foxp3 cells on graft survival was not established. In this respect, research in man is limited by ethical considerations related to sampling graft or lymph node tissue.

Another mechanism that may be involved in the tolerizing pretransplant blood transfusion effect is the occurrence of microchimerism, which is the persistence of a low number of donor-derived hematopoietic cells. The continuous presence of donor antigens may urge the recipient’s immune system into developing central and peripheral mechanisms to maintain tolerance for these antigens (2). In the NIMA setting, the importance of maternal cell microchimerism was already established. The presence of maternal hematopoietic cells in the child, due to bidirectional cell exchange during pregnancy (59), was associated with an improved transplant outcome of a NIMA-mismatched graft (60,61). In our study, we detected DNA of blood donor origin in only 1 out of seven PBT recipients two weeks after transfusion and in none at approximately 10 weeks after transfusion. The negative results at 2wk post-transfusion obtained in the other 6 patients do not exclude the existence of microchimeric cells, because the current techniques hold a risk of contamination and misannealing of the primers. In Chapter 6, a new technique that combines enrichment for donor HLA expressing cells by flowcytometry-based cell sorting and real-time PCR analysis is evaluated and proposed as a promising and valuable tool for future microchimerism detection.

Several factors may influence the effect of pretransplant blood transfusions, including the patient’s immune status at the moment of transfusion. We analyzed the effect of a pretransplant blood transfusion on the cellular immune system of two different patients groups that differed in previous exposure to HLA alloantigens. All DST
recipients were women who have been pregnant before, while pregnancy and therapeutic transfusions were exclusion criteria for PBT recipients. In many studies, exact data on the goal of giving a DST, e.g. in vivo cross match or tolerance-induction, and on patient selection criteria are lacking. In our study, HLA immunization and activation of cellular immunity occurred more frequently after DST compared to PBT. It is possible that the increased number and effector function of NK cells that was observed after DST, was influenced by the number of IFNγ producing T cells 2 wk post-DST. The difference in response towards PBT or DST is reflected in microarray analysis that showed a change in significantly more genes after DST than PBT, which is most likely the result of previous immunological priming to foreign HLA in the DST group. Another factor that may interfere with tolerance induction in transplantation is the immunosuppressive drug therapy that all transplant patients receive. Although we observed less severe rejection after pretransplant transfusion despite the use of potent immunosuppressive drugs (16), the drugs may destroy the recipient’s immune cells including tolerance-promoting Tregs. It is therefore essential to determine which immunosuppressive protocols block Tregs generation and which protocols do not. Cyclosporin A is reported to abrogate the generation of Tregs (62,63), whereas rapamycin is thought to save or even promote the Treg pool (64). Moreover, one has to take into account heterologous immunity that may be a barrier to tolerance induction (65). Memory cells that are elicited by certain viruses can enhance the clearance of unrelated pathogens (66). In the field of transplantation this could imply that virus-induced T cells may cross react with donor alloantigens and mediate graft rejection. It was shown in a mouse-model that heterologous immunity abrogated transplant tolerance that was established after DST in combination with anti-CD154 antibodies (67). Thus, differences in immune history may be the reason that tolerance does not develop in every patient following tolerance-inducing treatment.

Future perspectives of pretransplant blood transfusions
At the Leiden University Medical Center, two pretransplant transfusion policies existed. Pretransplant blood transfusions in the SPKT setting are associated with fewer patients with severe acute rejection episodes, as showed in chapter 2. The prevention of ATG rejection treatment after PBT diminishes the chance of developing immunosuppressive drug-related side effects. At the LUMC, the intention of the DST protocol was to act as an in vivo cross-match for women who have been pregnant. The decision of continuing the transplantation is based on the fact whether donor specific antibodies are induced by the DST. However, cellular reactivity was observed in almost every DST recipient as well and may hamper good graft function as well. Based upon a study in the Netherlands that showed a profound antibody induction after DST together with a lack of beneficial transplant outcome (68), the DST protocol was
abandoned in 2007. Apparently, hardly any confidence exists in a future application of pretransplant transfusions, as many centers abandoned the pretransplant blood transfusion policy. The missed opportunity to conduct well-designed RCTs and the lack of understanding the mechanisms contributed to this action.

The ultimate goal in clinical transplantation is the development of protocols that provide stable, long-term graft survival independent of immunosuppressive drug therapy. To be able to determine a state of tolerance in an individual patient that allows withdrawal of immunosuppressive drugs, reliable \textit{in vitro} assays are necessary that can predict B and T cell alloreactivity. However, standardized and sophisticated techniques are still lacking (69) and difficult to develop as confirmed by our \textit{in vitro} studies. Nevertheless, our investigations into the indirect allore cognition and microchimerism may be valuable for further research. A major point that supports the caution of transfusion allogeneic blood to individuals is the risk of developing alloantibodies that may hamper subsequent transplantation and the transmitting of infectious diseases (70). However, all blood products in the Netherlands are screened for many infectious agents and alloimmunization in our study was low.

This thesis started with the observation that fewer patients developed severe acute kidney graft rejection episodes in case they received a PBT before SPKT. This observation remained present in patients who received immunosuppressive induction therapy upon transplantation, which suggests that pretransplant blood transfusions may still be of benefit. However, to justify the pretransplant use of blood transfusions, well-designed RCTs are needed as well as reliable assays that can be used in the field of transplantation tolerance.
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47. van Twuyver E, Mooijaart RJ, ten Berge IJ et al. High-affinity cytotoxic T lymphocytes after non-HLA-sharing blood transfusion—the other side of the coin. Transplantation 1994;57: 1246-1251.


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