DIFFERENTIAL EFFECT OF PRETRANSPLANT BLOOD TRANSFUSIONS IN INDIVIDUALS WITH AND WITHOUT PREVIOUS HLA ALLOANTIGEN EXPOSURE


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Chapter 5

ABSTRACT

Pretransplant blood transfusions can improve graft function and survival after solid organ transplantation, but may also lead to activation of the immune response. The immunological mechanism leading to tolerance is not clear. We investigated the effect of a pretransplant leukocyte-containing blood transfusion on the phenotype and function of recipient lymphocyte subsets in individuals with previous exposure to HLA alloantigens by pregnancy and in individuals without previous HLA alloantigen exposure. In both patient groups, we detected an increase in the number of IFNγ producing T cells two weeks post-transfusion, reflecting activation of the recipient’s immune system. In patients with a prior exposure to HLA alloantigens, the increased frequency of IFNγ producing cells was also found longer after transfusion, as well as after third party stimulation. Moreover, in the pre-sensitized recipients blood transfusion led to an increased number of natural killer (NK) cells. Microarray analysis confirmed activation of NK cell-related genes and other immune genes exclusively in this patient group. In conclusion, blood transfusion led to activation of the immune system in both patient groups, while the effect on NK cells only occurred in prior HLA exposed individuals. Markers that indicate a state of tolerance were not detected, leaving the mechanism of the immunomodulating effect of pretransplant blood transfusions as yet unsolved.
INTRODUCTION

Allogeneic blood transfusions expose a recipient to many soluble and cell-bound antigens. In particular, contaminant leukocytes are presumed to play an important role in interacting with the recipient’s immune system. Blood transfusions can induce humoral immunity as reflected by the formation of HLA alloantibodies or, as opposite effect, suppress the recipient’s cellular immune system (1). An example of clinical benefit of this transfusion-induced immunosuppression is the improved graft outcome in patients who received a pretransplant blood transfusion (2,3). However, the mechanisms leading to organ donor-specific hyporesponsiveness after blood transfusion are not understood.

Because T cells play a central role in initiating an immune response towards the graft, it is possible that pretransplant blood transfusions may regulate these T cell responses ameliorating rejection and improve graft outcome. Several studies showed that allogeneic blood transfusion causes a shift in peripheral cytokine secretion by T helper (Th) cells from a Th1 phenotype towards that of a Th2 phenotype (1,4-6). Impairment of Th1 cytokine secretion may result in impairment of various functions of cellular immunity, including antigen processing, macrophage activation and cytotoxic T and natural killer (NK) cell functions. Storage of the blood product may contribute to immunoregulatory effects as well. It has been shown that soluble factors that arise in the blood product upon storage, like soluble HLA class I, class II and Fas ligand, can inhibit the mixed lymphocyte reaction (MLR) response and cytotoxic T lymphocyte (CTL) activity and induce apoptosis in Fas-expressing cells (7,8).

In experimental animal models, regulatory T cells (Tregs) that actively suppress alloreactive T cells responses have been demonstrated (9-11). The dual effect of blood transfusion, i.e. antibody induction at one hand and transplantation tolerance at the other is hypothesized to depend on the degree of HLA-DR sharing between transfusion donor and recipient (12). Complete DR-mismatched transfusions induced more often HLA antibodies than transfusions that are shared for one HLA-DR antigen with the recipient, while the latter may induce Tregs mediating organ donor hyporesponsiveness (13,14).

Our goal was to investigate the diverse effects of pretransplant blood transfusions on cellular immunity in two groups of recipients awaiting transplantation. We selected patients who received a pretransplant protocolled blood transfusion (PBT) before simultaneous pancreas-kidney transplantation and patients who received a donor-specific transfusion (DST) before living-related kidney transplantation. Patients with a history of pregnancy or prior therapeutic transfusion were excluded from PBT from a donor selected by sharing one HLA-DR antigen. In contrast, DST recipients were selected because of previous pregnancies and received a transfusion from their...
prospective kidney donor. This allows us to determine the impact of previous HLA exposure on the effect of a pretransplant blood transfusion.

MATERIALS AND METHODS

Recipients of a protocolled blood transfusion
Between September 2004 and August 2007, patients on the waiting list for simultaneous pancreas-kidney transplantation (SPKT) received a protocolled blood transfusion (PBT) at our center (Leiden, the Netherlands) if they had no history of blood transfusions or pregnancy. Patients were transfused with one unit of one-HLA-DR matched, non-leukocyte-depleted packed red blood cells, stored for less than 24 hours. The HLA phenotype of patients and donors was determined on a low resolution level by DNA-based typing using PCR-SSP. The presence of anti-HLA antibodies in patient’s sera before and after transfusion was determined by complement-dependent cytotoxicity. Based on the HLA antibody specificities detected in current and historical patient sera and the prevalence of HLA antigens in the specific donor population, a virtual PRA (panel reactive antibody) was calculated. It reflects the chance that a cross-match with a potential donor will be positive.

<table>
<thead>
<tr>
<th>Table 1: Characteristics of PBT recipients.</th>
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<td>Sex</td>
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P=patient, BD=blood donor. F=female, M=male.

Patient blood samples were collected before and after PBT (2 weeks and >10 weeks), and from each donor a blood sample was derived from the blood product. The collection of material was approved by hospital ethical committee. Peripheral blood mononuclear cells (PBMCs) from patient and PBT donor were isolated from heparinized blood by density gradient centrifugation with Ficoll-Amidotrizoate (Pharmacy LUMC, Leiden, the Netherlands). Cells were washed twice with phosphate-
buffered saline (PBS) and frozen in RPMI 1640 supplemented with L-glutamine (RPMI/glu, both from Gibco, Breda, the Netherlands), 20% fetal calf serum (FCS, Greiner, Alphen a/d Rijn, the Netherlands) and 20% dimethylsulfoxide (DMSO, Fluka, Buchs, Switzerland). Cells were frozen at -70°C and stored in liquid nitrogen until use. Third-party cells, with a similar number of mismatches as those between recipient and blood donor, were obtained from healthy volunteers. For *in vitro* analyses, we selected PBMCs of all patients with transfusion-induced HLA alloantibodies (except for one PBT recipient, due to lack of material). A random selection was made of the patients without HLA alloantibodies. Ten PBT recipients were selected for *in vitro* analyses. Their characteristics are shown in Table 1.

**Recipients of a donor-specific transfusion**
A donor-specific blood transfusion (DST) was given before living-related kidney transplantation in order to detect or boost historical sensitization. The selection of DSTs was independent of HLA phenotype, but equals the prospective kidney donor. Women with previous pregnancies were transfused with one unit of non-leukocyte-depleted, less than 24 hours stored packed red blood cells. Patient and donor PBMCs were collected and processed as described above. Fourteen DST recipients were selected for *in vitro* analyses, of whom half developed anti-HLA antibodies upon transfusion. Their characteristics are shown in Table 2.

**Table 2: Characteristics of DST recipients.**

<table>
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<tr>
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<th>Mismatches</th>
<th>Virtual PRA (%)</th>
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*P=patient, BD=blood donor. F=female, M=male.

*Mismatches:* HLA type: split level

*Virtual PRA:* 4 wk post-transfusion based on flowcytometric screening
Flow cytometry
For cell surface staining of lymphocyte subsets, activation markers, memory cell subsets and regulatory T cells (Tregs), thawed patient PBMCs were stained for 30 min at 4°C with fluorochrome-conjugated anti-human monoclonal antibodies (mAbs). General panel: anti-CD45 APC, anti-CD3 APC-Cy7, anti-CD4 PacB, anti-CD8 Amcyan, anti-CD14 PE-Cy7, anti-CD19 PE-Cy7, anti-CD56 AF700 and anti-γδTCR PE from BD Biosciences Pharmingen (San Diego, CA, USA). Activation panel: anti-CD3 PacB, anti-CD4 AF700, anti-CD8 FITC, anti-CD25 APC-Cy7, anti-CD28 APC, anti-CD14 PE-Cy7, anti-CD19 PE-Cy7 and anti-CTLA-4 PE-Cy5 (BD Biosciences Pharmingen), anti-HLA-DR PE-TxRed (Invitrogen, Eugene, OR, USA) and anti-CD127 PE (Beckman Coulter, Fullerton, CA, USA). Memory panel: anti-CD3 PacB, anti-CD4 APC-Cy7, anti-CD8 PE, anti-CD28 APC, anti-CD14 PE-Cy7 and anti-CD19 PE-Cy7 (BD Biosciences Pharmingen), anti-CD27 AF700 (eBioscience, San Diego, CA, USA), anti-CCR7 FITC (R&D systems, Minneapolis, MN, USA) and anti-CD45RA PE-TxRed (Invitrogen). Treg panel: anti-CD3 PacB, anti-CD4 AF700, anti-CD8 Amcyan, anti-CD25 APC-Cy7, anti-CD14 PE-Cy7 and anti-CD19 PE-Cy7 (BD Biosciences Pharmingen), anti-CD127 PE (Beckman Coulter), anti-CCR7 FITC (R&D systems) and anti-CD45RA PE-TxRed (Invitrogen) and anti-Foxp3 APC (eBioscience). mAbs were used in optimal titrated concentrations. Phenotypic characterization of CTLA-4 and Foxp3 required subsequent intracellular staining. To permeabilize cells for CTLA-4 staining, PBMC were incubated for 15 min with Intrastain fixative A (Glostrup, Denmark), washed, followed by 15 min incubation with fixative B and anti-CTLA-4 PE-Cy5. Intracellular staining for Foxp3 was performed using the APC-conjugated anti-Foxp3 Staining Set. A rat IgG2a isotype control (eBioscience) served as a negative control. To ensure correct measurement settings, compensation beads (uncoated and coated with anti-mouse IgG) stained with mAbs conjugated with each of the fluorochromes were used. Data were acquired using the BD LSR II flow cytometer (BD Biosciences) equipped with a 488nm laser, a 405nm laser, a 633nm laser and a 325nm to detect all the fluorescence parameters plus two scatter parameters. Data were analyzed using FACSDiva software (BD Biosciences).

Elispot assay
Reactivity of patient cells towards cells of the specific blood donor or third party was measured in the IFNγ enzyme-linked immunosorbent spot (Elispot) assay. First, 96-well Elispot plates (Millipore, Eschborn, Germany) were coated with a mouse anti-human IFNγ monoclonal antibody (Mab 1-D1K; Mabtech, Nacka Strand, Sweden) and left at 4°C overnight. Plates were blocked for 1 hour at 37°C with RPMI/glut containing 5% pooled human serum (HS). Responder PBMCs at a concentration of 1x10⁶ (in 100 μl) were stimulated with 2x10⁵ irradiated (4500 Rad) stimulator PBMC (in 50 μl) in triplicate wells in RPMI/glut containing 10% HS. Incubation was for 24h on an Elispot
Effect of pretransplant blood transfusion on recipient’s immune cells

plate (short assay) or for 4d in a 96-well round bottom plate (Costar®, Cambridge, MA, USA) followed by 24h on an Elispot plate (long assay). Medium and third-party donor cells (with a similar number of mismatches with the patient as the blood donor) were negative controls, whereas phytohemagglutinin (PHA, Remel Inc., Lenexa, KS, USA) served as a positive control. 48h and 4d after stimulation supernatant was collected for cytokine analysis.

After incubation (37°C, 5% CO₂) plates were washed with PBS supplemented with Tween20 and the anti-IFNγ detection antibody (Mab 7-B6-1-biotin; Mabtech, Nacka Strand, Sweden) was added for 2 hours at room temperature (RT). The conjugate (extravidin alkaline phosphatase, Sigma, St. Louis, MO, USA) was added for 1 hour at RT, followed by a washing step and addition of the BCIP/NBT substrate (Mabtech, Nacka Strand, Sweden) for 5 minutes at RT. The reaction was stopped with tap water. The resulting spots were counted automatically using the AID Elispot Reader System version 4.0 (AID, Strassberg, Germany).

Microarray analysis
Pre-transfusion and 2wk post-transfusion cryopreserved cells were thawed, washed twice in PBS and preserved in RNea later solution (Qiagen, Chatsworth, CA, USA). Total RNA was isolated from the samples using the RNeasy® mini Kit (Qiagen) following the manufacturer's instructions and treated with DNase (Qiagen) on the spin columns. RNA quantity was assessed with a spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) that determined the optical density (OD) at 260 nm. RNA quality was assessed using the Agilent RNA 6000 Nano LabChip® Kit with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Integrity of RNA was visualized by the 28S:18S ratio and the RNA integrity number (RIN). In our samples, the 28S:18S ratio, which ideally approaches 2, has a mean value of 1.9. A high RIN (scale from 0 to 10) is associated with less degradation and had a mean value of 7.9 in our samples. We used the Illumina® TotalPrep RNA Amplification Kit (Ambion, Austin, TX, USA) to amplify RNA for hybridization on Illumina BeadChips. To synthesize first strand cDNA by reverse transcription, we used 300-500 ng of total RNA from each sample. Following second strand cDNA synthesis and cDNA purification steps, the *in vitro* transcription for synthesis of cRNA was prepared overnight for 8h. The cRNA (1100 ng) of pre- and post-transfusion PBMCs was hybridized to Illumina HumanRef-8 v3.0 BeadChips (Illumina, San Diego, CA, USA), which contains over 24,000 genes. Gene expression profiles in pre- and post-transfusion samples were analyzed pair wise using the Illumina BeadStudio software. Only significantly expressed genes (9,606 in total) were selected for analysis.
Statistical analyses
The Wilcoxon signed rank test for matched pairs was performed to determine differences between pre- and post-transfusion cellular responses in Elispot and flowcytometry. Values of p<0.05 are considered to denote significant differences.

RESULTS

Phenotypic changes after transfusion
To determine whether pretransplant blood transfusions had caused a shift in different cell populations, pre- and post-transfusion (2wk and >10wk) recipient PBMCs were stained for cell surface and intracellular markers.

With regard to the activation or memory status of the cells, no significant changes were observed after a PBT or DST. Tregs, defined as CD4+CD25-Foxp3+CD127low cells, accounting for about 4% of the total CD3+ T cell population, were not affected by a pretransplant blood transfusion. However, the proportion of NK cells (CD3-CD56+) within the CD45+ cell population was altered after pretransplant blood transfusions, albeit in different directions dependent on whether the recipient was prior exposed to foreign HLA antigens or not (Figure 1). In PBT recipients the proportion of NK cells rather decreased, in particular after a longer interval (p=0.08), whereas in DST recipients this percentage increased significantly at 2wk post-transfusion (p=0.01).

Figure 1: Change in proportion of NK cells (CD3-CD56+) after PBT (A) or DST (B). Recipient pre-transfusion or post-transfusion (2wk or >10wk) PBMCs were thawed and labeled with anti-CD45, anti-CD3 and anti-CD56 monoclonal antibodies. Statistics: Wilcoxon matched pairs test. * P-value<0.05. PBMCs of 3 PBT recipients (PBT1, 2, 3) and 3 DST recipients (DST1, 5, 8) (black lines) were selected for microarray analysis.
IFN\(\gamma\) production by recipient cells

The effect of pretransplant blood transfusion on the functionality of recipient lymphocytes was measured by means of an IFN\(\gamma\) Elispot assay. To be able to measure the number of IFN\(\gamma\) producing cells, pre- and post-transfusion (2wk and >10wk) PBMCs of PBT and DST recipients were stimulated for 24h with blood donor (BD) PBMCs or third party (3P) PBMCs. As shown in Figure 2A, an increased number of IFN\(\gamma\) producing cells was found in PBT recipients after stimulation with the specific BD (p=0.02), but not after 3P stimulation (p=0.91). The increase in IFN\(\gamma\) producing cells had disappeared after a time-period longer than 10 weeks after PBT. As shown in Figure 2B, a similar reactivity of 2wk post-transfusion PBMCs of DST recipients was found (p=0.003). The IFN\(\gamma\) producing cells persisted more than ten weeks after DST (p=0.004). However, besides the increased number of IFN\(\gamma\) producing cells after specific stimulation, an increase was detected after third party stimulation (p=0.005 and p=0.004 for 2wk and >10wk respectively). We checked whether the NK cells were responsible for IFN\(\gamma\) production in the Elispot assay by performing a CD56+ depletion assay, but this was not the case (data not shown). The production of IFN\(\gamma\) was attributed to recipient T cells.

Naive T cell responses towards the specific blood donor, as determined in 5d Elispot, were not different in pre- and post-PBT or -DST samples (data not shown).

A. PBT recipients
Figure 2: IFN-γ Elispot of PBT recipients (A) and DST recipients (B). Recipient pre-transfusion or post-transfusion (2wk or >10wk) PBMCs were stimulated for 24h with irradiated blood donor PBMCs (BD) or third party PBMCs (3P) at a ratio of 1:2. The median number of spots is depicted by the horizontal line. Statistics: Wilcoxon matched pairs test. * P-value<0.05

Figure 3: Overview of fold change of all genes after PBT (n=3; y-axis) or DST (n=3; x-axis). Microarray analysis of a total of 9606 genes resulted in higher number of genes that were up-regulated or down-regulated after DST (472 and 206 respectively) compared with genes that were up-regulated or down-regulated after PBT (27 and 0 respectively).
Microarray analysis
To detect the influence of pretransplant blood transfusions on gene expression profiles, RNA of pre-transfusion and 2wk post-transfusion samples was analyzed using microarray analysis. RNA samples of PBT recipients 1, 2, 3 and DST recipients 1, 5, 8 showed good RNA quality and were selected for hybridization. A striking difference in fold change of genes after DST or PBT was observed. Figure 3 shows the difference in gene expression between pre-transfusion and 2wk post-transfusion samples analyzing 9606 genes. Many genes have been up- or down-regulated after DST, while gene expression is less affected in PBT recipients. A mean fold change in gene expression of 2-fold or more after DST resulted in 472 genes that were up-regulated and 206 genes that were down-regulated. In contrast, respectively 27 and 0 genes were at least 2-fold increased or decreased after PBT.

As the number of NK cells was increased in the DST group only, we specifically analyzed NK cell-related genes and identified 7 genes with a mean increase of at least 2-fold after DST. Expression of these genes remained unaltered, or even decreased in PBT recipients (Figure 4). The first three genes encode for the lytic proteins granzyme A/K and perforin, which are involved in the effector function of cytotoxic T cells and NK cells. The other genes (KLRF1, KIR2DL3, CD94 and CD160) encode for proteins expressed on NK cells only.

![Expression of NK cell-related genes](image_url)

Figure 4: Expression of NK cell-related genes in PBT (n=3) and DST recipients (n=3). Values are shown as mean ± sd. The dotted line represents the level of no change after transfusion (post/pre trf=1). KLRF1=killer cell lectin-like receptor member 1, KIR2DL3=killer-cell immunoglobulin-like receptor 2DL3.
Chapter 5

DISCUSSION

The goal of this study was to gain more insight into the effect of pretransplant blood transfusions on the recipient’s cellular immune system. Pretransplant blood transfusions can cause immune activation as well as immune suppression, but the exact immunological mechanisms of these effects are still unsolved. The present study shows that both a PBT and DST activate the recipient’s immune system as reflected by an increased number of IFNγ-producing cells 2 weeks post-transfusion. This is in agreement with previous studies that observed an increased frequency of helper and cytotoxic T cells (HTlp and CTLp respectively) (15,16), irrespective of the degree of HLA matching between recipient and blood donor (17,18). However, contradictory results are also reported, such as a decrease in CTLs after a HLA-shared blood transfusion (19,20) or no change at all (21). The question why T cell activation after a first stimulus by blood transfusions would lead to hyporesponsiveness towards a subsequent organ donor has been addressed before. Terasaki (22) hypothesized that the graft acts as a second stimulus and concurrent immunosuppressive drug treatment particularly deletes these reactivated T cells. This could indeed explain why increased HLTp and CTLp frequencies are associated with better graft survival. However, this cannot explain the difference on graft survival between HLA-DR shared and non-shared transfusions.

We investigated whether Tregs are activated after a pretransplant blood transfusion. It has been hypothesized that especially after HLA-DR shared blood transfusion Tregs may play an important role (13). This was supported by experimental animal models, in which Tregs were generated after DST, able to transfer tolerance to naïve mice (10,23). Tregs (CD4+CD25+Foxp3+CD127low) in our patient population, accounted for about 4% of CD3+ cells, but their proportion was not changed shortly or long after PBT or DST. Moreover, when we analyzed intracellular and cell surface activation markers, there was no change in the number of activated cells, whereas a higher activity of blood donor-specific recipient T cells was found 2 weeks post-transfusion by Elispot analysis. This suggests that while the percentage of activated cells remains the same, their effector function may be increased. The contribution of Tregs may be missed during in vitro investigation of peripheral blood cells. Encounter of alloantigens and initiation of the immune response occurs in lymphoid organs, which is the presumed site for immune regulatory mechanisms to take place. Increased numbers of Tregs were found in lymph nodes as well as in the graft upon transplantation (11). Moreover, a second boost by the graft may be necessary for sufficient Treg numbers to develop (24). Our phenotypic analyses of recipient PBMCs do not give any indication on the role of Tregs to explain a beneficial blood transfusion effect, but rather suggest activation of T helper 1 cells.
The investigation of PBT recipients as well as DST recipients provides information about the effect of former HLA alloantigen exposure on blood transfusion outcome. HLA antibody formation occurred more frequently after DST than after PBT. Additionally, after DST recipient T cells were affected in a broader way. In contrast to PBT recipients, DST recipients not only showed an increased number of IFNγ producing T cells for a longer interval after transfusion, but also against a third party estimated in the Elispot assay. This profound T cell activation may account for the increased number of NK cells (CD3-CD56+) that we observed after DST in phenotypic analysis. CD4+ T cells can stimulate NK cells via cytokines, such as IFNγ, encouraging the NK cells to obtain effector functions. NK cells are cells of the innate immune system that can kill target cells through the release of lytic proteins, such as perforin and granzymes. They express activating and inhibitory surface molecules that interact with specific HLA class I molecules on the target cells. The profound T cell activation may account for this effect.

Microarray analysis showed that, indeed, there was an increased expression in DST recipients of genes that encode for proteins involved in the cytolytic process by NK cells, such as perforin, granzyme A, and K. There was also an increased expression of the receptors KLRF1 or NKp80 (an activating receptor) and KIR2DL3, an inhibitory receptor. Genes encoding for CD94 and CD160, predominantly expressed on NK cells, were increased as well. The effect on NK cells may be the result of missing self antigens on the blood donor leukocytes as each blood donor expresses an HLA class I ligand (HLA-C or Bw4) for the receptors on recipient NK cells. However, PBT donors also express missing self antigens, showing that this hypothesis cannot account for the difference in NK cell activation between PBT and DST recipients. Previous studies that investigated the effect of blood transfusions on NK cells, reported an impaired NK cell reactivity, similar as we observed after PBT.

Not only NK cell-related genes were up- and down-regulated after DST, but many other genes, a phenomenon not observed after PBT. It is likely that these differences between PBT and DST recipients in gene expression profiles reflect the primary and memory immune responses respectively. As all DST recipients were primed for HLA alloantigens by pregnancy, T cells of DST recipients may respond in an accelerated and heightened manner towards alloantigens as compared to PBT recipients. Other factors that may influence the difference between PBT and DST recipients are the underlying disease and HLA compatibility between recipient and blood donor. All PBT recipients had developed end-stage renal disease caused by type I diabetes mellitus, which was not the case in DST recipients. It is not likely that differences in HLA compatibility are of notice. Patients who received a DST matched for one HLA-DR antigen (n=8), thus comparable with PBT recipients, showed similar findings in Elispot and phenotypic analysis compared with the whole group (n=14). In microarray, all 3 PBT and DST recipients were matched for 1 HLA-DR antigen with the blood donor.
In conclusion, PBTs as well as DSTs activated the recipient’s T cell compartment, whereas DSTs also activated NK cells. While clinical transplantation outcome is improved in patients who received a PBT (29), we were not able to detect a parameter for transplantation tolerance in these patients.
Effect of pretransplant blood transfusion on recipient’s immune cells

References