Donor-Reactive T-cell Responses after HLA-Identical Living-Related Kidney Transplantation

Jeroen H. Gerrits

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Donor-Reactive T-cell Responses after HLA-Identical Living-Related Kidney Transplantation

Donor-gerichte T-cel reactiviteit na HLA-identieke familieniertransplantatie

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Voor

Sandra

Ik heb je lief,
ik heb je liever,
liever dan mijn leven,
dan om het even wat.
Ik heb je lief,
ik heb je liever,
liever, liefste, elke dag.

(Stef Bos, 'ik heb je lief')

en

Lars

Je hebt zoveel
gezien vandaag.
Alles is nog vreemd.
Zo veel kleuren,
geluiden
waar je de namen niet van weet.

(Stef Bos, 'Slaapliedje')

Contents

Chapter 1

General Introduction

Jeroen H. Gerrits, Willem Weimar, and Nicole M. van Besouw

Based on Minerva Urol Nefrol 2007;59(3):367-377

1. KIDNEY TRANSPLANTATION

Kidney transplantation is the preferred treatment of choice for almost all categories of patients with end-stage-renal disease (ESRD) including those with hypertension, glomerulonephritis, diabetes mellitus and genetic causes as polycystic renal disease. Transplanted patients will live approximately 10-15 years longer than patients that remain on dialysis. In light of the increased donor shortage and the increasing waiting time, the number of kidney transplants from living donors enormously increased during the last decade. Complete matching for major Human Leucocyte Antigen (HLA) molecules between donor and recipients is preferable, but due to the high degree of polymorphism of HLA, only some recipients with a suitable living-related sibling donor receive a fully HLA-identical donor kidney.[1]

1.1 HLA-mismatched kidney transplantation

After HLA-mismatched deceased or living kidney donation, the primary targets of the immune response of the transplant recipient are the major HLA molecules present on the donor kidney. Mismatches in major HLA molecules between donor and recipient may provoke strong immune responses resulting in graft rejection when not treated with immunosuppressive medication.[2]

Allorecognition may occur via the direct or indirect recognition pathway (Figure 1).[2, 3] The direct allorecognition pathway involves recipient T cells recognising intact major HLA molecules presented by donor antigen presenting cells (APC). The most potent APC are dendritic cells (DC).[4] The indirect allorecognition pathway refers to the recognition of recipient T cells recognising peptides from donor origin presented by self-HLA molecules on recipient APC. In both recognition pathways, recipient $CD4^+$ and $CD8^+$ T cells may contribute to graft rejection. The frequency of responding T cells is high in the direct allorecognition pathway, whereas in the indirect allorecognition pathway the frequency of responding T cells is low.[5] The direct allorecognition pathway plays an important role in the early phase after organ transplantation, and is thought to play a significant role during acute rejection. Donor cells migrate to recipient lymph nodes for presentation to recipient T cells.[2, 6] The indirect allorecognition pathway plays a role in the late phase after transplantation, and it has been proposed to be responsible for the development of chronic allograft rejection.[2, 7]

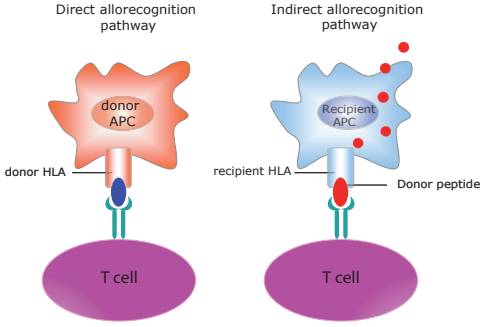

Figure 1: Direct and indirect allorecognition pathway after kidney transplantation. During direct allorecognition, recipient T cells recognize intact HLA molecules presented by donor antigen presenting cells (APC). During indirect recognition, recipient T cells recognize donor peptides after they have been processed and presented by self-HLA molecules on the surface of recipient APC.

1.2 HLA-identical living-related kidney transplantation

After HLA-identical living-related (LR) kidney transplantation, all major HLA molecules (HLA-A, B, C, DR, DP and DQ) from recipient and donor are identical, only mismatches in non-HLA antigens, like minor histocompatibility antigens (mHAgs) may exist. mHAgs are genetic inherited peptides derived from polymorphic intracellular proteins presented in the context of HLA class I and II molecules, which can be recognized by recipient T cells.[8] In humans, the number of identified mHAgs has been expanded, reaching 17 autosomally encoded mHAgs and 10 Y-chromosome encoded antigens (Table 1).[9, 10] The expression of mHAgs is broad or restricted, *e.g.* specific for proximal tubular epithelial cells of the kidney or haematopoietic cells.[8, 10] In contrast to the strong immune responses directed to major HLA molecules, responses to mHAgs do not provoke strong immune responses in cellular tests.[8]

In humans, the clinical relevance of mHAgs has almost exclusively been shown after bone marrow transplantation (BMT).[10] The first indication that mHAgs could be important for transplant outcome was observed in a female patient, who had aplastic anaemia, transplanted with bone marrow from her HLA-identical brother. This patient had cytotoxic T-lymphocytes (CTL), which recognised structures restricted to male cells encoded by the structures on the Y chromosome.[11, 12]

The clinical relevance of mHAgs after solid organ transplantation is yet not clear. After HLA-identical LR kidney transplantation, mismatches in mHAgs[13] and other non-HLA antigens[14] between donor and recipient may induce an immune response that may result into rejection of the donor kidney. Krishnan *et al.*[15] reported an association between HA-1 mismatches in HLA-A2 matched donor-recipient pairs and chronic allograft nephropathy (CAN) evidenced by increased graft failure.In contrast, Heinold *et al.*[16] reported that mismatches in mHAgs between donor and recipient had no influence on the 5-years graft survival.

One report showed that mHAgs could be relevant after HLA-identical LR sibling kidney transplantation. Despite treatment of azathioprine (AZA) and prednisone, a female patient who received a donor kidney from her HLA-identical brother, experienced acute rejection within 3 weeks.[17] In this report was demonstrated in cell-mediated lympholysis (CML) that cytotoxic T cells from the patient were directed to male-specific HY mHAg.

We showed that donor immune reactivity can also be determined by IFN-γ Elispot assay in the first period after HLA-identical LR kidney transplantation.[18]

1.3 Organ allograft rejection and immunosuppressive medication

Renal allograft rejection can be classified into hyperacute rejection, acute rejection, and CAN. Hyperacute rejection occurs rapidly within 24 hours after transplantation due to pre-existing humoral antibodies against donor cells, while acute rejection may occur in the first three months after kidney transplantation.[19] CAN is a main cause of late renal failure and limits the long-term outcome of organ transplantation.[20] Clinically, CAN is characterised by a decrease in renal function and the development of proteinuria, but also chronic damage, *e.g.* atherosclerosis, glomerulosclerosis, interstitial fibrosis and tubular atrophy, demonstrated in renal biopsies.[20, 21]

To prevent graft rejection, transplant recipients receive immunosuppressive medication to suppress the immune response directed to the foreign organ. In 1954, the first kidney transplantation was performed between HLA-identical twins to prevent problems of an immune rejection.[22] The first successful kidney transplantation between non-twins

Table 1: Human minor histocompatibility antigens (mHAgs)

mHAgs	HLA-restriction	Gene	Chromosomal location	Immunogenetic peptide	Allelic counterpart	Distribution
HA-1	HLA-A2/B60	*HA-1*	19	VL**H**DOLLEA	VLRDDLLEA	Restricted
HA-2	HLA-A2	*Myosin 1G*	7	YIGEVLVS**V**	YIGEVLVSM	Restricted
HA-3	HLA-A1	*LBC Oncogene*	15	V**T**EPGTAQY	VMEPGTAQY	Broad
HA-8	HLA-A2	*KIAA0020*	9	**R**TLDKVLEV	PTLDKVLEV	Broad
HB-1H	HLA-B44	*Unknown*	5	EEKRGSL**H**VW	EEKRGSLYVW	Restricted
HB-1Y	HLA-B44	*Unknown*	5	EEKRGSL**Y**VW	EEKRGSLHVW	Restricted
ACC-1	HLA-A24	*BCL2A1*	15	DYLQ**Y**VLQI	DYLQCVLQI	Restricted
ACC-2	HLA-B44	*BCL2A1*	15	KEFED**D**IINW	KEFEDGIINW	Restricted
SP110 (HwA-9)	HLA-A3	*SP110*	2	SLP**R**GTSTPK	SLPGGTSTPK	Restricted
PANE-1 (HwA-10)	HLA-A3	*CENPM*	22	**R**VWDLPGVLK	*VWDLPGVLK	Restricted
UGT2B17	HLA-A29/B44	*UGT2B17*	4	AELLNPFLY	Gene deletion	Restricted
LRH-1	HLA-B7	*P2x5*	17	TPNQRQNVC	Nucleotide insertion	Restricted
ECGF-1	HLA-B7	*ECGF1*	22	RP**H**AIRRPLAL	RPRAIRRPLAL	Restricted
CTSH	HLA-A31	*Cathepsin H*	15	ATLPLLCA**R**	ATLPLLCAG	Restricted
CTSH	HLA-A33	*Cathepsin H*	15	WATLPLLCA**R**	WATLPLLCAG	Restricted
LB-ADIR-1F	HLA-A2	*ADIR*	1	SVAPALAL**F**PA	SVAPALALSPA	Restricted
ACC-6	HLA-B44	*HMSD*	18	MEIFIEVFSHF	Exon deletion	Restricted
HY	HLA-A1	*USP9Y*	Y	IVDCLTEMY	IVDSLTEMY	Broad
HY	HLA-A2	*SMCY*	Y	FDSYICQV		Broad
HY	HLA-A33	*TMSB4Y*	Y	EVLLRPGLHFR		Broad
HY	HLA-B52	*RPS4Y1*	Y	TIRYPDPVI		Restricted
HY	HLA-B60	*UTY*	Y	RESEEESVSL		Broad
HY	HLA-B7	*SMCY*	Y	SPSVDKARAEL		Broad
HY	HLA-B8	*UTY*	Y	LPHNHTDL		Restricted
HY	DQB1*05	*D0X3Y (DBY)*	Y	HIENFSDIDMGE		Broad
HY	DRB1*1501	*D0X3Y (DBY)*	Y	SKGRYIPPHLR		Broad
HY	DRB3*0301	*RPS4Y1*	Y	VIKVNDTVQI		Broad

Adapted from Goulmy *et al.*[10], department of Immunohaematology and Blood Bank, Leiden, The Netherlands and *www.lumc.nl/5033/dbminor/index.asp?page=antigen/antigen.asp.*

was performed five years later.[23] The latter recipient received total body radiation pre-transplantation to decrease the strength of the immune system.

Nowadays, kidney transplantation is the preferred renal replacement therapy. Patients with chronic renal failure who have a living donor available are able to receive a renal transplant before dialysis is needed. In our transplant center, the 1-, 5- and 10-years unadjusted (not censored for death) renal graft survival is 78%, 56% and 39%, respectively, in deceased donors. The graft survival at 1-, 5- and 10-years in recipients of LR not HLA-identical donor is 89%, 78% and 61%, respectively, and is 93%, 84% and 70%, respectively, in recipients of a LR HLA-identical sibling donors (Figure 2).

The development of immunosuppressive drugs, to inhibit the reaction of the immune system, improved the prevention and treatment of acute rejection.[24] For the last 50 years, corticosteroids and azathioprine (AZA) have been used in organ transplantation as immunosuppressive medication. Corticosteroids influences genes involving in priming the innate immune response, suppresses pro-inflammatory cytokines and promote anti-inflammatory cytokines.[25] AZA inhibits the proliferation of activated immune cells by interfering with DNA synthesis.[25, 26] In the early 1980s, cyclosporine A (CsA) was introduced as a new immunosuppressive drug, and the number of acute rejections significantly decreased. CsA is a calcineurin inhibitor (CNI), which binds to cyclophilin resulting into inactive calcineurin. Consequently, calcineurin is unable to dephosphorylate nuclear factor of activated T cells (NFAT), and therefore, IL-2 production is prevented.[25, 26] In the last decade, also other immunosuppressive agents became available, including tacrolimus (another CNI), mycophenolate mofetil (MMF), sirolimus and everolimus, daclizumab and basiliximab, and leflunomide.[25, 26]

Despite successful treatment of transplant recipients with immunosuppressive drugs to prevent allograft rejection, life-long administration of immunosuppressive drugs after transplantation is deemed necessary. However, long-term use of immunosuppression is associated with severe side-effects, such as malignancies, infections, nephrotoxicity, hypertension, diabetes mellitus and osteoporosis.[26-28] Consequently, tapering or withdrawal of immunosuppression is warranted, provided this is not accompanied with transplant rejection. Therefore, it is important to identify patients in whom the immunosuppressive load can be tapered or even withdrawn to reduce the side-effects of immunosuppression.

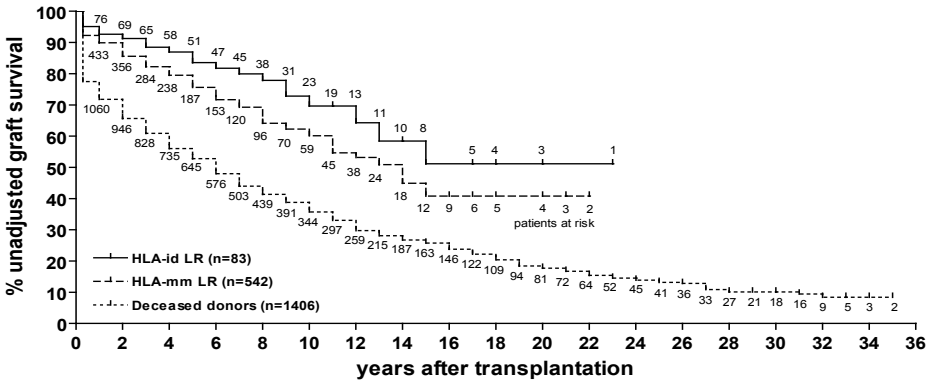

Figure 2: The unadjusted (not censored for death) graft survival after kidney transplantation in Erasmus Medical Center, Rotterdam, The Netherlands. Recipients of a deceased donor kidney are presented as dotted line, recipients of a living-related not HLA-identical donor kidney are presented as dashed line (HLA-mm LR), and recipients of a living-related HLA-identical sibling donor kidney are presented as solid line (HLA-id LR).

2. IMMUNE MONITORING BEFORE AND AFTER KIDNEY TRANSPLANTATION

Monitoring for the presence of immunological activity directed to the graft is necessary to identify patients in whom the immunosuppressive medication can be safely tapered. Biopsies taken from the donor kidney may provide valuable information. Unfortunately, this is an invasive technique and unpleasant for the transplant recipient. Therefore, it should be avoided for routine diagnoses. Immune monitoring in peripheral blood would be preferred.

There are several non-invasive assays using PBMC to determine immune reactivity to donor antigens, e.g. mixed lymphocyte reaction (MLR), cell-mediated lympholysis (CML), CTL precursor frequency (CTLpf), helper T-lymphocyte precursor frequency (HTLpf), Elispot assay, flow cytometry, tetramer staining, measurement of donor-specific alloantibodies and cytokine mRNA quantification.[29] Here, the focus was only on donor-specific assays in PBMC: proliferative capacity of PBMC in MLR, cytotoxic and helper reactivity in CTLpf and HTLpf, respectively, and the frequency of cytokine producing cell in Elispot assay.

In an one-way mixed lymphocyte culture or MLR, the proliferative capacity of recipient cells to donor cells is determined. Using CML, the capacity of recipients' cytotoxic T-lymphocytes (CTL) to kill donor cells can be determined. The number of recipients' CTL responding to donor antigens can be measured in limiting dilution assays (LDA).[30-32] The supernatant of the LDA in combination with an IL-2-dependent murine CTLL-2 cell line can be used to determine the number of helper T-lymphocytes (HTLpf).[33-35] The Elispot assay (Enzyme-Linked ImmunoSpot assay) is designed to enumerate cytokine secreting cells directed to donor cells.[18, 36]

2.1 Immune monitoring of alloreactivity before kidney transplantation

Pretransplant determination of HLA and anti-HLA antibodies [panel-reactive antibodies (PRA) and cross match] has been in practice during the last 4 decennia to prevent hyperacute rejection.

The percentage PRA is the percentage of cells from a panel of blood donors against which potential recipients' serum reacts. The higher the PRA, the more sensitized a patient is to the general donor pool, and thus more difficult to find a suitable donor. When a potential suitable donor is available, a cross match is performed to detect possible preformed antibodies in a potential recipients' blood against antigens on the surface of a potential donor cells. In our center, both current and historic serum had to be cross match negative with the potential donor cells to prevent hyperacute rejection after renal transplantation.

Before kidney transplantation it is important to identify those patients who are at risk for future allograft rejection.[30, 35-41] Poggio *et al.*[38] and Andree *et al.*[41] described the humoral response in combination with the cellular response as screening method before transplantation. The cellular response was based on T-cell alloreactivity to a panel of allogeneic stimulator PBMC from healthy individuals by analysing the number of IFN-γ producing cells using Elispot assays, creating a Panel of Reactive T-cell (PRT) assay. The percentage PRT is the percentage of stimulators that stimulated memory T cells, *i.e.* IFN-γ producing cells. Haemodialysis patients had higher PRT values than healthy individuals.[41] T-cell alloreactivity based on PRT can be independent of positive PRA, and vice versa.[38] Increased number of patients have to be tested to assess the pretransplant risk for acute rejection by PRT.

Several studies demonstrated that pretransplant CTLpf[30, 35, 42] and MLR[42] failed to predict the clinical outcome after renal transplantation. Nevertheless, we demonstrated that both high HTLpf and MLR determined before kidney transplantation were predictive

for patients who developed acute rejection in the first year after kidney transplantation.[35] Patients with low or undetectable donor-specific HTLpf and MLR remained free from acute rejection.[35]

Also, the number of IFN-γ producing cells determined by Elispot assays has been correlated with posttransplantation outcomes.[36, 37, 39, 43] Increased pretransplant frequency of donor-specific IFN-γ producing cells was related with posttransplant risk for acute rejections[36, 39], and impaired posttransplant renal function.[37]

2.2 Immune monitoring of alloreactivity after tapering or withdrawal of immunosuppression in HLA-mismatched kidney transplant recipients

After kidney transplantation, patients receive a maintenance dose of immunosuppression to prevent graft rejection. Chronic use of immunosuppression is associated with severe side-effects.[26-28] Therefore, nowadays, an important goal after kidney transplantation is to identify those patients in whom the immunosuppressive load can be safely reduced. Most of these studies were performed in the HLA-mismatched kidney transplant setting.[31, 32, 44-56]

Some studies described the withdrawal or avoidance of steroids after kidney transplantation.[48, 49] Other studies reported good results by withdrawal of CNI[45] or converting from CNI to AZA or MMF[50-53], or halving the AZA or MMF dose.[47, 54]

Most of the studies did not correlate the ability of successfully tapering the immunosuppressive drugs with *in vitro* immune function. Some studies evaluated the MLR and others the CML assay.[45, 55, 56] However, because of small number of patients, a limited prognostic factor was found between MLR or CML reactivity of patients' cells to donor antigens and acute graft rejection. A study from Beik *et al.*[44] suggested that donor-specific hyporeactivity was associated with a lower incidence of acute rejection after steroid withdrawal in a small cohort of kidney transplant recipients.

Our group studied stable renal transplant recipients, who were routinely converted from CsA and prednisone to either AZA or MMF in combination with prednisone at 1-year after kidney transplantation. Thereafter, the AZA or MMF dose was tapered to 50% of their original dose.[31, 46, 51] Clinically, conversion of CsA to AZA or MMF improved serum creatinine levels. After conversion, less acute rejection occurred in the MMF group (4/34) compared to the AZA group (11/30). Tapering of AZA or MMF was possible in most of the patients, only three acute rejections occurred in both groups during tapering of AZA (3/25) or MMF (3/26).[51] We demonstrated that donor-specific CTLpf determined before conversion to AZA or MMF was higher in patients who experienced acute rejection after conversion compared to patients who remained free from acute rejection (Figure 3), while no difference was observed in third-party reactivity. No relation was found with tetanus toxoid responses, donor-specific and third-party responses in MLR and HTLpf. Because a donor-specific CTLpf of <10 CTL/10^6 PBMC had a positive predictive value of 100% for not developing rejection after conversion from CsA to AZA or MMF, we suggest that renal transplant recipients with no or low numbers of donor-specific CTLpf could be safely tapered in their immunosuppressive load.[31] Subsequently, the T-cell reactivity was determined before and after tapering of AZA or MMF.[46] Donor and third-party reactivity determined in MLR, CTLpf and HTLpf was not affected after tapering of AZA or MMF, while tetanus toxoid reactivity was increased.

Recently, we showed that withdrawal of CNI in kidney transplant recipients at least 2 years after kidney transplantation was associated with a significant decrease in donor-specific CTLpf, while third-party reactive CTLpf, donor and third-party reactive MLR and IFN-γ Elispot remained unaffected. Moreover, the percentage of $CD4^+CD25^{bright}CTLA\text{-}4^+$ T cells (regulatory

T cells) was increased.[32] Regulatory cells have the ability to regulate alloresponses. These cells may also regulate effector CTLp. Therefore, we speculate that CNI inhibits regulatory mechanism that could downregulate donor responses. Consequently, after CNI withdrawal, the immunosuppression could be tapered further. Thereafter, we indeed demonstrated that patients, who had donor-specific CTLpf below 10/10^6 PBMC, can be tapered to 50% of their AZA or MMF dose without affecting their serum creatinine.[47, 54]

Nowadays, changes in number of circulating regulatory T cells are a hype in models of tolerance. An increased number of $CD4^+CD25^{high}$ in tolerant liver transplant recipients is reported.[57] In small cohort of kidney transplant recipients it was shown that clinically tolerant patients did not differ in their number of $CD4^+CD25^{high}$ from healthy individuals, while chronic allograft nephropathy is associated with a decrease in $CD4^+CD25^{high}$.[58] To our knowledge controlled studies on tolerance and regulatory cells are not yet described.

The group of Soulillou[59] showed that five tolerant kidney transplant patients have a for transplant recipients unique blood T-cell repertoire of T-cell receptor Vβ and CDR3 usage compared to patients with CAN and stable patients with immunosuppression. This assay using polymerase chain reaction (PCR) and TcLandscape analyses does not need donor cells, but the importance of this assay had to be confirmed in larger series.

DC are APC that may initiate and regulate immune responses.[4] DC can be divided into myeloid DC (mDC) and plasmacytoid DC (pDC). mDC produce high levels of IL-12 and induce T-helper 1 and CTL responses. In contrast, pDC produce IFN-α and seems to play a role induction of T-helper 2 responses.[60] Moreover, Kuwana *et al.*[61] suggested that pDC can be involved in the induction of peripheral T-cell tolerance after organ transplantation. Several studies showed that immunosuppression in transplant recipients influence DC subset numbers.[62-64] After liver transplantation, patients who were completely off immunosuppression had higher pDC/mDC ratio compared to patients on maintenance therapy.[65] These results indicate that without using donor cells tolerant transplant recipients can be identified.

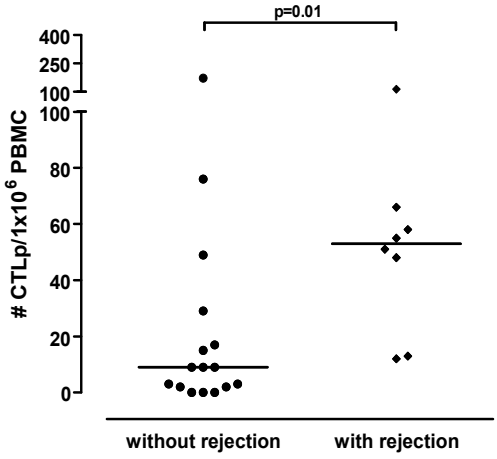

Figure 3: Frequencies of donor-specific CTLp before conversion of CsA to AZA or MMF from patients who remained free from acute rejection and from patients who experienced acute rejection after conversion. The significance of difference was calculated with the Mann-Whitney U-test.

2.3 Immune monitoring after HLA-identical LR kidney transplantation

In the HLA-identical LR kidney transplant setting, no donor-reactive T-cell responses directed to non-HLA antigens, *i.e.* mHAgs, can be determined by MLR, CTLpf and HTLpf.[18] However, in the IFN-γ Elispot assay significant responses were found after stimulation with donor cells.[18] Recently, we found that HLA-identical LR kidney transplant recipients had significantly lower numbers of donor-reactive IFN-γ producing cells two years after transplantation than at a stable period 3 months after transplantation (Figure 4). However, some patients had detectable numbers of donor-reactive IFN-γ producing cells even long after kidney transplantation. No literature is known about the necessity of long-term use of immunosuppression in HLA-identical LR kidney transplant recipients, and whether withdrawal of immunosuppression influenced donor-reactive T-cell responses.

2.4 Immune monitoring of chronic rejection

The long-term outcome after kidney transplantation remains still a problem due to CAN[20]. It has been suggested that the indirect recognition pathway plays a role in CAN.[2, 20]

A report from Ciubotariu *et al.*[66] showed that the indirect recognition pathway contributes to the development of coronary artery vasculopathy (CAV) in heart transplant recipients. Hornick *et al.*[67] could detect in 5 out of 7 heart transplant recipients with CAV, elevated frequencies of T cells with indirect anti-donor specificity, while no indirect anti-donor reactivity was detected in patients without CAV. Recently, we found increased donor-reactivity via the indirect recognition pathway during late acute rejection episodes after clinical heart transplantation.[68]

Baker *et al.*[69] showed in renal transplant recipients (with and without CAN), long after kidney transplantation, donor-specific hyporesponsiveness via the direct recognition pathway according to proliferation, CTLpf and cytokine secretion of IFN-γ, IL-2 and IL-5. Remarkable was that anti-donor proliferative response via the indirect recognition pathway was higher in patients with CAN compared to patients without CAN. Recently, Poggio *et al.*[7] reported that renal transplant recipients with CAN had higher IFN-γ Elispot responses compared to those without CAN. These results suggest that the indirect recognition pathway play is a relevant tool to monitor late acute rejection and chronic rejection long after organ transplantation.

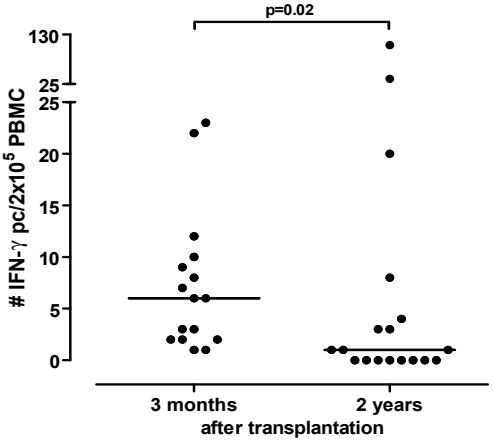

Figure 4: Number of donor-reactive IFN-γ producing cell (pc) determined in PBMC from HLA-identical living-related kidney transplant recipients 3 months after kidney transplantation and 2 years after kidney transplantation. The Mann-Whitney U-test was used to compare the patient groups.

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Chapter 2

Aim and outline of the thesis

The aim of this thesis is to clarify the clinical relevance of donor-reactive T-cell responses directed to minor histocompatibility antigens (mHAgs) after HLA-identical living-related (LR) kidney transplantation. The thesis is divided in two parts: (1) 'Donor-reactive T-cell responses after HLA-identical LR kidney transplantation' and (2) 'Tapering of immunosuppression after HLA-identical LR kidney transplantation'.

In **Chapter 1** we describe non-invasive cell mediated immune assays to determine donor-reactive T-cell responses in peripheral blood from transplant recipients taken before transplantation and during tapering or withdrawal of immunosuppression. Those donor-reactive T-cell responses could be helpful to identify transplant recipients in whom the immunosuppressive load can be safely reduced.

After HLA-identical sibling donor bone marrow transplantation (BMT), graft-versus-host disease may occur which is likely due to alloantigens known as mHAgs.[1] However, the clinical relevance of mHAgs after HLA-identical LR kidney transplantation is yet unknown. Given the existence of possibly hundreds of mHAgs[2], it is reasonable to suggest that transplant outcome might be influenced by mHAg disparities between HLA-identical donor-recipient pairs. Therefore, theoretically, if immune reactivity occurs after HLA-identical LR kidney transplantation, it should be directed to mismatched mHAgs or other non-HLA antigens present on the donor kidney.

It has been reported that donor-reactive T-cell responses may decrease during the first year after HLA-mismatched organ transplantation.[3-7] **Chapter 3** describes whether donor-reactive IFN-γ and granzyme B (GrB) producing cells also decreases during the first year after HLA-identical LR kidney transplantation. Donor-recipient couples were typed for 11 known mHAgs in an endeavour to relate the number of known mHAg mismatches between donor and recipient with the number of donor-reactive cytokine producing cells. **Chapter 4** studies the number of donor-reactive cytokine producing cells (IFN-γ, IL-10, GrB, IL-13) after HLA-identical LR kidney transplantation and after HLA-mismatched LR kidney transplantation to investigate whether donor reactivity in the HLA-identical setting was different from the HLA-matched setting during immunological quiescence. Healthy individuals served as controls. **Chapter 5** describes the expression profile of 42 cytokines in donor-stimulated mixed lymphocyte cultures from peripheral blood mononuclear cells (PBMC) of HLA-identical LR kidney transplant recipients using a novel protein array.

Monitoring of myeloid dendritic cells (mDC) and plasmacytoid DC (pDC) in peripheral blood following liver transplantation might identify patients in whom the immunosuppressive dose can be safely tapered.[8] Since it is less time consuming and more accurate to measure sequential blood samples of one patient in one flow cytometric session, we examined the effect of cryopreservation of PBMC from kidney transplant recipients on mDC and pDC percentages and the expression of maturation markers in comparison to fresh blood cells as described in **Chapter 6**. In addition, PBMC from kidney transplant recipients were compared with heart failure patients, who received no immunosuppressive medication, to study the influence of immunosuppression on the DC subsets.

In general, recipients of an HLA-identical LR kidney transplant receive the same immunosuppressive medication as those of an HLA-mismatched kidney transplant. Because the necessity of long-term use of immunosuppression in HLA-identical LR kidney transplant recipients is yet unknown, we questioned whether these patients could be tapered in their immunosuppressive load despite mismatches in mHAgs between donor and recipient. Theoretically, tapering or withdrawal of immunosuppression in organ transplant recipients may

result into increased alloreactivity.[9-12] Therefore, immunological monitoring of donor reactivity in those patients could be helpful to identify patients in whom the immunosuppressive load can be safely reduced or even withdrawal. Previously, we demonstrated that donor reactivity can be determined after HLA-identical LR kidney transplantation.[13]

Chapter 7 and 8 studies the effect of halving the azathioprine (AZA) dose in HLA-identical LR kidney transplant recipients on renal function and on T-cell reactivity determined by the number of IFN-γ, IL-13 and GrB producing cells against donor, 3rd-party and tetanus toxoid.

Thereafter, in patients on mycophenolate mofetil (MMF) or AZA in combination with prednisolone therapy the AZA and MMF dose was withdrawal. **Chapter 9** describes the clinical results after discontinuation of AZA or MMF and the two years follow-up of these patients on prednisolone monotherapy. In the same patient group, the effect of discontinuation of MMF and AZA on T-cell reactivity and DC subsets is described in **Chapter 10**. T-cell reactivity was determined using IFN-γ, IL-10 and GrB Elispot assays, and circulating DC subset numbers and their maturation status were determined by flow cytometry. In **Chapter 11**, the effect of MMF or AZA withdrawal on 17 soluble cytokines from supernatant of donor-stimulated mixed lymphocyte reactions determined by multiplex bead array technique was analysed.

Finally, the results described in this thesis are summarized and discussed in **Chapter 12**.

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Part I

Donor-Reactive T-cell Responses after HLA-Identical Living-Related Kidney Transplantation

Chapter 3

Non-HLA reactivity during the first year after HLA-identical living-related kidney transplantation

Non-HLA reactivity after kidney transplantation

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Abstract

Background: It has been reported that donor-reactive T-cell responses may decrease during the first year after HLA-mismatched organ transplantation. We wondered whether donor-reactive T-cell responses directed to minor histocompatibility antigens (mHAgs) or other non-HLA antigens also decrease after HLA-identical living-related (LR) kidney transplantation.

Methods: We studied donor-reactive T-cell responses by IFN-γ and granzyme B (GrB) Elispot assays in 15 HLA-identical LR kidney transplant recipients before, 6-months and one year after transplantation. Third-party reactivity was used as control. Patient and donor peripheral blood mononuclear cells were typed for 11 known mHAgs.

Results: During the study period, 60% and 36% of the patients demonstrated donor-reactive IFN-γ and GrB producing cells (pc), respectively. The number of donor-reactive IFN-γ and GrB pc was significantly lower than the number of third-party reactive IFN-γ and GrB pc. After transplantation, donor-reactivity and third-party reactivity were comparable to pretransplant values. No relation was found in mHAg mismatches between donor and recipient and donor-reactive T-cell response.

Conclusions: Donor-reactivity could be detected before and after HLA-identical LR kidney transplantation, but was not related with the number of mHAg mismatches, and did not decrease after transplantation.

INTRODUCTION

Kidney transplantation is the preferred treatment of choice for patients with end-stage-renal disease. In case of kidney transplantation, complete matching for major HLA molecules between donor and recipients is preferable. However, due to the high degree of polymorphism of HLA, only recipients with a suitable living-related (LR) sibling donor can receive a fully HLA-identical donor kidney.[1]

Apart from major HLA molecules, also other antigens may elicit an immune response after transplantation, for example, minor histocompatibility antigens (mHAgs). mHAgs are peptides derived from polymorphic proteins which can be presented in HLA class I or II molecules and are inherited independently of HLA.[2] In humans, the clinical relevance of mHAgs has been shown after bone marrow transplantation (BMT).[3] Therefore, theoretically, immune responses after HLA-identical LR kidney transplantation could be directed to the mismatched mHAgs or other non-HLA antigens. Indeed, a role of non-HLA antigens was suggested after solid organ transplantation.[4, 5]

After HLA-mismatched organ transplantation, several studies reported a decrease in donor-reactive T-cell response during the first year after kidney, liver and lung transplantation using mixed lymphocyte culture (MLC), cytotoxic T-lymphocyte precursor frequency (CTLpf), helper T-lymphocyte precursor frequency (HTLpf) and IFN-γ Elispot assays.[6-10]

Recently, we demonstrated that donor-reactive responses can be found after HLA-identical LR kidney transplantation.[11] In the present study, we questioned whether in these patients donor-reactive cytokine responses may also decrease within the first year after transplantation. Therefore, we used IFN-γ and granzyme B (GrB) Elispot assays, which reflect the Th1 response and the activity of cytotoxic T lymphocytes (CTL), respectively.

PATIENTS AND METHODS

HLA-identical living-related (LR) kidney transplant recipients

The Ethical Review Committee of our center approved the protocol, which was conducted according to local requirements. After informed consent, 35 ml peripheral blood was taken from 15 HLA-identical LR kidney transplant recipients before transplantation, 6-months and one year after transplantation, and from their related donors. The characteristics of the patient group are described in Table 1.

Peripheral blood mononuclear cell sampling

Peripheral blood mononuclear cell (PBMC) from both recipient and donor was isolated from heparinized blood by density gradient centrifugation using Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden). PBMC were collected from the interphase, washed twice with RPMI-1640-DM (Cambrex, Verviers, Belgium) supplemented with 100 IU/ml of penicillin (Cambrex) and 100 µg/ml of streptomycin (Cambrex). PBMC were stored in RPMI-1640-DM containing 15% foetal calf serum (FCS) and 10% dimethyl sulphoxide (MERCK, Hohenbrunn, Germany) at –140°C until use.

Table 1: Demographics of the HLA-identical LR kidney transplant recipients

Patient	Gender				Yrs on	PRA	Immunosuppressive therapy after transplantation (mg/day) (6-mo)					Immunosuppressive therapy after transplantation (mg/day) (one year)					Time of recurrence
	R	D	#Tx	RRT	RRT	(%)	Tacro	MMF	Pred	AZA	CsA	Tacro	MMF	Pred	AZA	Primary disease	after Tx (yrs)
1	M	M	1	CAPD	1.3	0	4	1000				4	1000			Focal segmental glomerulosclerosis	No
2	F	M	1	HD	4.0	0			5	125				2.5	100	AL-amyloïdose	No
3	M	F	1	HD	1.3	4	6	500				6	500			IgA nephropathy	No
4	M	F	2	HD	13.0	4	10	1500				10	1000			Focal segmental glomerulosclerosis	No
5	F	F	2	HD	1.1	41		500	2.5							Membranous proliferative glomerulonephritis	No
6	M	F	1	CAPD	0.1	0	4	2000				4	1500			Membranous proliferative glomerulonephritis	1.0
7	M	F	1	HD	4.0	0		1000	5				1000	5		Polycystic kidney disease	No
8	F	F	1	CAPD	1.5	78	6	1500				6	1500			Membranous proliferative glomerulonephritis	2.4
9	F	M	1	None	0	0	10	2000	20			10	2000	20		Polycystic kidney disease	No
10	M	M	1	CAPD	1.0	6			10		400			10	175	IgA nephropathy	No
11	M	M	1	CAPD	1.0	0	4	1000				3				Polycystic kidney disease	No
12	M	F	1	HD	1.4	0	4	750	5			6	500			Focal segmental glomerulosclerosis	0.3
13	M	F	1	None	0	0	2	1000				2	1000	5		Pauci-immune glomerulonephritis	No
14	F	F	1	None	0	0	5	1000				2	500			Tubular interstitial nephritis	No
15	F	F	1	CAPD	1.0	0		1000	5							Hypertension	No
median			1		1.3	0	5	1000	5			5	1000	7.5	137.5		

R, recipient; D, donor; M, male; F, female; #Tx, number of kidney transplantations; RRT, renal replacement therapy before kidney transplantation; CAPD, continuous ambulatory peritoneal dialysis; HD, haemodialysis; PRA, panel reactive antibodies before transplantation; mo, months; Tacro, tacrolimus; MMF, mycophenolate mofetil; Pred, prednisone; AZA, azathioprine; CsA, cyclosporine A; -, no immunosuppressive medication.

IFN-γ and GrB Elispot assays

The viability of PBMC was tested in the phytohemagglutinin (PHA; Murex Biotech, Kent, UK) proliferation assay as described before.[11] The stimulation index (SI) was calculated by the ratio of the counts per minute (cpm) obtained in the presence of PHA to the cpm in the absence of PHA. Only data of viable cells (SI ≥ 50) are presented.

The donor-reactive T-cell response was determined using IFN-γ and GrB Elispot assays (U-CyTech biosciences, Utrecht, The Netherlands) as described before.[11, 12] In a 96-wells round bottom plate (Nunc, Roskilde, Denmark), six replicates of $2x10^5$ patients' PBMC were preincubated with 100 µL of $2x10^5$ irradiated (40 Gy) donor PBMC, irradiated patients' PBMC (autologous response used as negative control to control the influence of irradiation), or third-party PBMC, which were completely HLA-mismatched with donor and recipient. After 40 h of incubation, the non-adherent cells were harvested and resuspended in culture medium. The non-adherent cells were transferred in triplicate to a flat-bottom plate (Nunc, Roskilde, Denmark) precoated with IFN-γ or GrB monoclonal antibodies (U-CyTech biosciences) and post-coated with phosphate-buffered saline (PBS) supplemented with blocking stock solution B (U-CyTech biosciences). Cells were incubated for 5 hours at 37°C at the IFN-γ and GrB Elispot plate. After incubation, the cells were lysed with ice-cold milli-Q water and washed extensively. Subsequently, the wells were incubated overnight at 4°C with 100 µL of diluted biotinylated goat anti-human cytokine (IFN-γ or GrB) polyclonal antibody (U-CyTech biosciences) followed by incubation with 50 µL phi-labelled goat anti-biotine antibodies (U-CyTech biosciences) for 1 hour at 37°C. After washing the wells, 30

μL of reagent (activator I + II, U-CyTech biosciences), that activates phi, was added and incubated for 15 to 30 minutes at room temperature in the dark. The reaction was stopped by adding milli-Q water to the wells. The spots were counted automatically by using a Bioreader 3000 Elispot reader (BioSys, GmbH, Karben, Germany).

The autologous response was subtracted from the donor and third-party response. The autologous response was significantly lower than the donor-reactive response (IFN-γ, p=0.01; GrB, p=0.04).

mHAgs typing after HLA-identical LR kidney transplantation

Patient and donor PBMC were typed for 11 known mHAgs: HA-1, HA-2, HA-3, HA-8, HB-1, ACC-1, ACC-2, HwA-9, HwA-10, UGT2B17, and HY. DNA from donor and recipient were isolated using the QIAamp® DNA Mini Kit.[13] The PCR-SSP (sequence specific primers) technique was used as typing method. This is a PCR based technique with the use of SSPs, which shortens the post-amplification processing time to a simple gel electrophoresis detection step. Each tested mHAgs is part of a di-allelic system. This means that each locus contains two alleles, and a specific primer combination for each allele was used to test the presence of this allele.

Statistical analysis

The Wilcoxon signed rank test was used to compare the number of IFN-γ pc and GrB pc before, 6-months and one year after HLA-identical LR kidney transplantation. The Spearman's rank correlation coefficient (r_s) was used to correlate the number of IFN-γ pc and GrB pc reactive to donor and third-party cells. Two sided P-values ≤0.05 were considered significant. For statistical analysis, SPSS 11.5 for Windows was used (SPSS, Inc., Chicago, IL, USA).

RESULTS

Clinical results

At one year after HLA-identical LR kidney transplantation, 12 from the 15 patients had stable serum creatinine levels (median, 98 μmol/L; range, 68-139) and no proteinuria (<0.5 g/L). Patient 6, 8 and 12 developed recurrence of their original disease, membranous proliferative glomerulonephritis (MPGN) and focal segmental glomerulosclerosis (FSGS), at 1.0, 2.4 and 0.3 years after transplantation, respectively (Table 1). None of the 15 patients experienced acute rejection episodes, or infections with cytomegalovirus or Epstein-Barr virus.

The frequency of IFN-γ and GrB producing cells

We found in 53% (8/15), 60% (9/15) and 67% (10/15) of the patients detectable (≥1 cytokine producing cells (pc)/2x10^5 PBMC) donor-reactive IFN-γ pc before, 6-months and one year after transplantation, respectively (Table 2). Before transplantation (median, 2 IFN-γ pc/2x10^5 PBMC; range, 0-129), the number of donor-reactive IFN-γ producing cells (pc) was comparable to the number of IFN-γ pc at 6-months (median, 1 IFN-γ pc/2x10^5 PBMC; range, 0-58; p=0.75) and one year (median, 2 IFN-γ pc/2x10^5 PBMC; range, 0-64; p=0.31; Figure 1A) after transplantation. No change was observed in the reactivity against third-

party antigens before transplantation (median, 67 IFN-γ pc/2x10^5 PBMC; range, 0-283) and after transplantation (6-months: median, 86 IFN-γ pc/2x10^5 PBMC; range, 0-166; p=0.28; one year: median, 82 IFN-γ pc/2x10^5; range, 3-173; p=0.94; Figure 1B). The donor-reactive response was at all time points significantly lower than the third-party reactive response (before, p=0.001; 6-months, p=0.001; one year, p=0.002).

From 13 of the 15 HLA-identical patients, we were able to perform GrB Elispot assays. In 31% (4/13) of the patients detectable responses to donor antigens were found before transplantation, and 38% (5/13) of the patients had detectable responses both at 6-months and one year after transplantation (Table 2). The number of donor-reactive GrB pc was comparable before transplantation (median, 0 GrB pc/2x10^5 PBMC; range, 0-192), 6-months (median, 0 GrB pc/2x10^5 PBMC; range, 0-131; p=0.73) and one year (median, 0 GrB pc/2x10^5 PBMC; range, 0-54; p=0.69; Figure 1C) after transplantation. The third-party reactive response before transplantation (median, 76 GrB pc/2x10^5 PBMC; range, 3-231) was comparable with 6-months (median, 56 GrB pc/2x10^5 PBMC; range, 4-177; p=0.58) and one year (median, 33 GrB pc/2x10^5 PBMC; range, 0-151; p=0.21; Figure 1D) after transplantation. At all time points, the donor-reactivity was always lower compared to the third-party reactivity (before, p=0.001; 6-months, p=0.001; one year, p=0.01).

Overall, the number of donor-reactive and third-party reactive IFN-γ pc correlated with the number of donor-reactive and third-party reactive GrB pc (donor: r_s=0.71; n=39; p<0.0001; third-party: r_s=0.55; n=39; p=0.0003; Figure 2A and B).

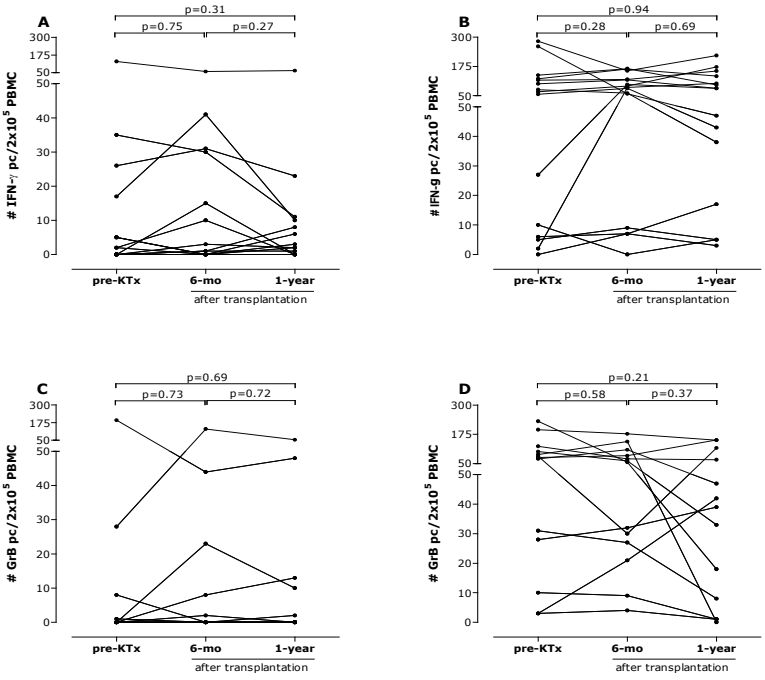

Figure 1: The number of IFN-γ (n=15) and granzyme B (GrB) (n=13) producing cells reactive to donor cells (A, C) and third-party cells (B, D) before, 6-months (mo) and 1-year after HLA-identical living-related kidney transplantation. (KTx, kidney transplantation)

Relation between the number of donor-reactive cytokine pc and known mHAg mismatches after HLA-identical LR kidney transplantation

We analysed whether donor-reactive responses (≥1 cytokine pc/2x10^5 PBMC) found after HLA-identical LR kidney transplantation were directed to known mismatched mHAgs between donor and recipient (Table 2).

From the 15 patient-donor combinations, nine couples (patient 1, 2, 6, 7, 8, 9, 11, 12, 14) demonstrated at least one mismatch for known mHAgs between donor and recipient. Seven out of nine couples had a mHAg mismatch in combination with the correct HLA-restriction molecule in which the donor mHAgs could be presented to T cells. Those seven patient-donor combinations demonstrated no differences in cytokine producing cells with the other eight patient-donor combinations (IFN-γ: p=0.54; GrB: p=0.75; Fischer's Exact test)

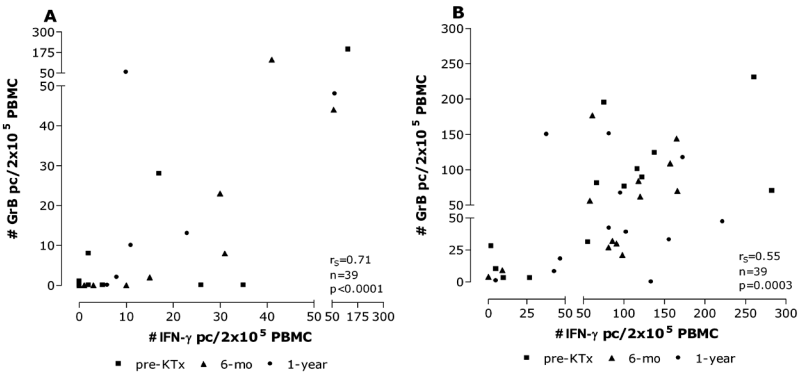

Figure 2: Correlation between the number of IFN-γ (n=39) and granzyme (GrB) (n=39) producing cells reactive to donor cells (A) or third-party cells (B) (squares, pretransplant values; triangles, 6-mo values; circles, 1-year values).

DISCUSSION

After HLA-identical LR kidney transplantation, immune responses could be directed to the mismatched mHAgs or other non-HLA antigens between donor and recipient. In contrast to immune responses against major HLA molecules, mHAgs do not induce strong immune responses in cellular assays.[14] In our study, donor-reactive cytokine responses (≥1 cytokine pc/2x10^5 PBMC) could be detected even before and after HLA-identical LR kidney transplantation. Those donor-reactive T-cell responses before transplantation could be a result of paternal or maternal mHAg priming of fetal T cells.[15] They could provide a continuous source of those mHAgs that may maintain during adult life.[16]

During the study period, we found that 60% (27/45) and 36% (14/39) of the PBMC samples responded to donor antigens in IFN-γ and GrB Elispot assays, respectively. The frequency of donor-reactive IFN-γ pc and GrB pc in PBMC from stable HLA-identical LR kidney transplant recipients in our study was comparable with the frequency of donor-reactive IFN-γ pc and GrB pc in PBMC from stable HLA-mismatched kidney transplant recipients in other

Table 2: Known mismatched minor histocompatibility antigens and donor-reactive IFN-γ and granzyme B producing cells after HLA-identical LR kidney transplantation

Patient	Mismatched mHAgs between donor and recipient	HLA-restriction molecule present	IFN-γ (cytokine pc/2x10^5 PBMC)			GrB[1] (cytokine pc/2x10^5 PBMC)		
			before	6-mo	1-year	before	6-mo	1-year
1	HA-2M		0	1	8	0	0	2
2	*HB-1H, HY*[3]	B44, DQ5	5	0	6	0	0	0
3	no difference		35	30	11	0	23	10
4	no difference		26	31	23	0	8	13
5	no difference		2	10	0	0	0	0
6	HA-1R		2	0	2	8	0	0
7	HA-2M, ACC1-Y, ACC2-D, *HwA-9R*, HwA-10	A3	0	1	1	nd[2]	nd	nd
8	*HA-8R*	A2	17	41	10	28	131	54
9	HA-1H, HY	A2, DQ5	5	0	0	0	0	0
10	no difference		0	0	3	nd	nd	nd
11	*HwA-9R*	A3	0	3	2	0	0	0
12	*HA-1H*	B60(40)	129	58	64	192	44	48
13	no difference		0	0	0	1	0	0
14	*HA-1H*, *HA-2V*, *HA-8R*, ACC-1Y, *ACC-2D*, HwA-9G, HwA-10	A2, B44	0	15	0	0	2	0
15	no difference		0	0	0	0	0	0

[1]GrB, Granzyme B; [2]nd, not enough cells were available to perform also GrB Elispot assays; [3]Mismatched mHAgs with the known HLA-restriction molecule are presented as cursive.

studies, who were 3 to 18 months after transplantation.[17-19] Additionally, the number of donor-reactive GrB pc from our previous study were comparable with the number of donor-reactive GrB pc at 6-months (p=0.83) and one year (p=0.83) after transplantation.[20] Thus, low numbers of donor-reactive IFN-γ pc and GrB pc can be found during immunological quiescence and are independent from HLA matching.

The clinical relevance of mHAgs in HLA-identical LR kidney transplantation is yet unknown. A study from Krishnan *et al.*[21] reported in a small cohort of patients an association between HA-1 mismatch and chronic allograft nephropathy. In contrast to this study, we found no relation between known mHAg mismatches in combination with the correct HLA-restriction molecule and the number of donor-reactive cytokine producing cells. In our previous studies, we were also unable to find an association between known mHAgs mismatches and donor-reactive responses.[20, 22] Additionally, Heinold *et al.*[23] reported that mHAg mismatches between donor and recipient after cadaveric and living-related kidney transplantation had no significant influence on the five years death-censored allograft survival.

Several studies reported a decrease in T-cell response during the first year after organ transplantation.[6-10] To our knowledge, there are no reports describing donor-reactive cytokine responses during the first year after HLA-identical LR renal transplantation. In the present study, we found that the numbers of donor-reactive and third-party reactive IFN-γ pc and GrB pc in PBMC from HLA-identical LR kidney transplant recipients remained stable during the first year after transplantation. The number of donor-reactive IFN-γ pc increased in some patients and decreased in others in the first six months after transplantation, while no

further increase was found at one year after transplantation. These results were comparable with our previous data.[11] Apparently, during immunological quiescence, reactivity directed to non-HLA antigens do not have to be actively downregulated. Therefore, no decrease in donor-reactive IFN-γ pc and GrB pc was observed.

In the HLA-mismatched setting, studies demonstrated that pre- and posttransplant numbers of IFN-γ pc were associated with impaired serum creatinine levels.[18, 24] In the present study, we found no correlation between pre- and posttransplant numbers of cytokine pc and serum creatinine levels. Recent studies suggested that use of dialysis and even time on dialysis results in higher numbers of alloreactive IFN-γ pc compared to short-term use of dialysis.[25, 26] In our study, we observed no relation between the number of cytokine pc and time on renal replacement therapy. We also found no relation between the number of cytokine pc and PRA in HLA-identical LR kidney transplant recipients.

In the present study, still 67% and 38% of the patients had detectable donor-reactive responses in IFN-γ and GrB Elispot assays, respectively, at one year after transplantation. Remarkably, in five HLA-identical LR kidney transplant recipients donor-reactivity was not detectable in both IFN-γ and GrB Elispot assays at one year after transplantation. We suggest that HLA-identical LR kidney transplant recipients without any detectable donor-reactive IFN-γ pc and GrB pc should already be tapered in their immunosuppressive medication within the first year posttransplantation. This is probably too early for those patients with detectable numbers of donor-reactive cytokine producing cells. In some reports a small number of HLA-identical LR kidney transplant recipients are described during complete discontinuation of immunosuppression with stable graft function.[27-29]

It has been suggested that natural killer (NK) cells could also play a role in solid organ transplantation.[30, 31] NK cells are able to produce pro-inflammatory cytokines as IFN-γ and GrB.[31, 32] However, in our Elispot assays, no difference in response was found between PBMC depleted for NK cells and PBMC not depleted for NK cells (data not shown). Therefore, we assume that NK cells play not a role in our test system.

In conclusion, in peripheral blood, non-HLA reactivity can be detected before and after HLA-identical LR kidney transplantation. The height of the donor-reactivity was not related with the number of known mHAgs mismatches. We observed no decrease in reactivity against those donor antigens during the first year after HLA-identical LR kidney transplantation. In 67% of HLA-identical LR kidney transplant recipients, donor-reactivity is detectable even at one year after transplantation, and in 33% of the patients no donor-reactivity was found.

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Chapter 4

Donor-reactive cytokine-profiles after HLA-identical living-related kidney transplantation

Cytokine-profiles after HLA-identical living-related kidney transplantation

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ABSTRACT

Background: After HLA-identical living-related (LR) kidney transplantation, only non-HLA antigen mismatches between donor and recipient may exist. We questioned whether donor-reactive responses against non-HLA antigens could be found after HLA-identical LR kidney transplantation, and wondered whether donor-reactivity in the HLA-identical setting was different from the HLA-mismatched setting during immunological quiescence. Healthy individuals served as controls.

Methods: Elispot assays were performed to determine the number of alloreactive IFN-γ producing cells (pc), IL-10 pc, granzyme B (GrB) pc and IL-13 pc from peripheral blood mononuclear cells (PBMC) of HLA-identical, HLA-mismatched LR kidney transplant recipients and healthy individuals.

Results: The frequency of alloreactive IFN-γ pc, IL-13 pc and GrB pc was higher in healthy individuals compared to both transplant patient groups. In the HLA-identical group, significantly higher numbers of donor-reactive IL-10 pc were found compared to their autologous control. These frequencies were also higher compared to the HLA-mismatched and healthy control group. The number of donor-reactive GrB pc was higher in the HLA-mismatched group than in the HLA-identical group. Donor-reactive IFN-γ pc and IL-13 pc were comparable in both transplant groups.

Conclusions: In recipients of HLA-identical LR kidney transplant, high donor-reactive IL-10 pc, in combination with low donor-reactive IFN-γ pc, IL-13 pc and GrB pc, suggests active downregulation of reactivity against non-HLA molecules.

INTRODUCTION

After HLA-identical LR kidney transplantation, all major HLA molecules are identical (HLA-A, B, C, DR, DQ) with the donor, and only mismatches may exist in non-HLA antigens or minor histocompatibility antigens (mHAgs). Minor HAgs are HLA-restricted peptides derived from cellular proteins that differ in amino acid sequence between donor and recipient due to genetic polymorphisms.[1-3] After HLA-identical allogeneic bone marrow transplantation (BMT), immune responses are caused by mHAg mismatches between donor and recipient.[3] Theoretically, after HLA-identical LR kidney transplantation, both mismatches in mHAgs[4] and mismatches in other non-HLA antigens[5], may induce rejection of the kidney transplant.

Cytokines are important mediators in regulating lymphocyte activation, proliferation, differentiation, and survival.[6] Pro-inflammatory cytokines, such as interleukin (IL)-2, interferon (IFN)-γ and tumor necrosis factor (TNF)-α, have been postulated to promote allograft rejection. In contrast, anti-inflammatory cytokines, such as IL-4, IL-5, IL-10 and IL-13, have been associated with downregulation of the immune response.[7-9] Some studies also reported that anti-inflammatory cytokines, such as IL-4, were present during rejection after clinical heart transplantation.[10, 11]

Recently, studies reported a beneficial role for IL-10 after BMT and after HLA-mismatched kidney transplantation.[12-15] After BMT with HLA-identical sibling donors, donor-stimulated peripheral blood mononuclear cells (PBMC) showed significantly higher numbers of IL-10 producing cells (pc) in Elispot assays and higher levels of IL-10 mRNA expression levels in the absence of graft-versus-host disease (GVHD) compared to samples taken during GVHD.[13, 14] After HLA-mismatched kidney transplantation, higher numbers of donor-reactive IL-10 pc in PBMC were found by Elispot assays in the absence of rejection compared to during acute rejection.[15] In addition, high levels of IL-10 determined in supernatants from unstimulated PBMC by enzyme-linked immunosorbent assay (ELISA) was associated with stable graft function.[12]

Cytotoxic T-lymphocytes (CTL) play a crucial role in allograft rejection.[16, 17] CTLs are capable of inducing apoptotic cell death via the death receptor pathway, such as FAS-FAS ligand, or via the exocytosis pathway, which involves perforin and granzyme B (GrB). The number of CTL responding to donor antigens can be measured by determining the CTL precursor frequency (CTLpf) in limiting dilution assays.[18] After HLA-identical LR kidney transplantation, no donor-reactive responses can be measured by CTLpf. An alternative for cytotoxicity is to determine the activity of CTL by using the IFN-γ or GrB Elispot assay.[19-21]

In the present study, we questioned whether we could detect donor-reactivity against non-major HLA molecules after HLA-identical LR kidney transplantation. Autologous responses were used as negative controls. Furthermore, we wondered whether the donor-reactive response in the HLA-identical setting was different from the HLA-mismatched setting during immunological quiescence.

SUBJECTS AND METHODS

Kidney transplant recipients and healthy individuals

Both the transplant recipients and kidney donors visited our out clinic regularly to control their renal function. After informed consent, 35 ml heparinized blood was taken from both recipients and their specific donors of a HLA-identical (n=13) and HLA-mismatched (n=12) LR

kidney transplants, and healthy individuals (n=10: n=5 male and n=5 female blood donors). The characteristics of HLA-identical and HLA-mismatched LR kidney transplant recipients are described in Table 1A and B, respectively. We included 10 healthy individuals (n=5 male and n=5 female blood donors). From the HLA-identical recipients, one patient received his second graft and two patients their third graft (Table 1A). In the HLA-mismatched group, two patients received their second graft. After HLA-identical LR kidney transplantation, none of the patients experienced an acute rejection episode. In the HLA-mismatched group, only one patient developed an acute rejection period within 1 week after transplantation (Table 1B).

Peripheral Blood Mononuclear Cells (PBMC) sampling

PBMC from transplant recipients, their specific kidney donors and healthy individuals were isolated from heparinized blood by density gradient centrifugation using Ficoll-Paque (density 1.0777 g/ml; Amersham Biosciences, Uppsala, Sweden). PBMC were collected from the interphase, washed twice with RPMI-1640-DM (Cambrex, Verviers, Belgium) supplemented with 100 IU/ml of penicillin (Cambrex) and 100 µg/ml of streptomycin (Cambrex). Thereafter, PBMC were stored in RPMI-1640-DM containing 15% foetal calf serum (FCS) and 10% dimethyl sulfoxide (MERCK, Germany) at −140°C until use.

Elispot assay: IFN-γ, IL-10, IL-13, and granzyme B (GrB)

The phytohemagglutinin (PHA) proliferation assay was performed to control the viability of the PBMC as described before.[18] The stimulation index (SI) was calculated by the ratio of the cpm obtained in the presence of PHA to the cpm in the absence of PHA. Only results of viable cells (SI ≥ 50) were analyzed in the described results.

Alloreactivity was determined in an IFN-γ, IL-10, IL-13 and GrB Elispot assays. In a 96-well round bottom plate (Nunc, Roskilde, Denmark), six replicates of $1x10^5$ PBMC from healthy individuals were stimulated with 100 µl of $1x10^5$ irradiated (40 Gy) PBMC derived from another healthy individual. In the HLA-mismatched setting, $1x10^5$ patients' PBMC were stimulated with 100 µl of $1x10^5$ irradiated (40 Gy) donor-specific PBMC. Because we expected low frequencies of cytokine pc in the HLA-identical setting, 100 µl of $2x10^5$ patients' PBMC was added to 100 µl of $2x10^5$ irradiated donor-specific PBMC to increase the sensitivity of the Elispot assays. Additionally, irradiated stimulator cells alone were incubated in culture media [if cytokine producing cells (pc) were detected, <5 spots/$2x10^5$ PBMC in IFN-γ, IL-13 and GrB Elispot assays, and <20 spots/$2x10^5$ PBMC in IL-10 Elispot assays were found]. To control the influence of irradiation on cytokine production, responder cells were incubated with irradiated responder (40 Gy) cells (autologous response). The autologous response was substracted from the alloresponse. Responder PMBC in the culture medium alone were used as a negative control (Figure 1A-D). As a positive control, PBMC were stimulated with 1 µg/ml PHA (Murex Biotech, Kent, UK) (Figure 1A-D). After 40 h of incubation at 37°C and 5% CO_2, the non-adherent cells were collected, washed, and resuspended in 300 µl culture medium. The non-adherent cells were transferred in 3 wells of a flat-bottom 96-well plate (Nunc, Roskilde, Denmark) pre-coated with either mouse anti-human IFN-γ, IL-10, IL-13 or GrB monoclonal antibody (U-CyTech Biosciences, Utrecht, The Netherlands) and post-coated with phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA; U-CyTech Biosciences). The cells were incubated for 5 hours at 37°C and 5% CO_2 for IFN-γ, IL-13 and GrB Elispot assays, and overnight for IL-10 Elispot assay. After incubation,

Table 1A: Characteristics of HLA-identical living-related kidney transplant recipients[a]

	Gender		Age (years)	Time after KTx[e] (years)	PRA[f] (%)	Immunosuppresive regimen (mg/day)				
ID[b]	Pat[c]	Don[d]				CsA[g]	Tacro[h]	MMF[i]	AZA[j]	Pred[k]
1	Female	Male	35	2.7	2				50	7.5
2	Male	Female	54	2.1	2		4	1500		
3	Female	Female	58	1.0	27			500		10
4	Male	Female	45	0.5	37	350				10
5	Male	Male	49	0.3	5	450				15
6	Female	Male	32	6.4	83					7.5
7	Male	Female	30	0.6	31	300				10
8	Male	Male	29	3.5	0				200	10
9	Male	Female	53	0.5	2	400				10
10	Female	Male	48	1.0	0				50	2.5
11	Male	Male	49	0.9	0		4			5
12	Male	Female	54	0.9	0		4	1000		
13	Male	Female	58	0.8	0		2	1000		5
		median	49	0.9	2	375	4	1000	125	10

[a]HLA-identical LR kidney transplantation, identical at the HLA-A, B, C, DR, and DQ loci; [b]ID, identification number; [c]Pat, patient; [d]Don, donor; [e]KTx, kidney transplantation; [f]PRA, panel-reactive antibodies; [g]CsA, cyclosporine A; [h]Tacro, tacrolimus; [i]MMF, mycophenolate mofetil; [j]AZA, azathioprine; [k]Pred, prednisone.

Table 1B: Characteristics of HLA-mismatched living-related kidney transplant recipients

	Gender		Age (years)	Time after KTx[d] (years)	PRA[e] (%)	HLA-mm[f]	Immunosuppresive regimen (mg/day)				
ID[a]	Pat[b]	Don[c]					CsA[g]	Tacro[h]	MMF[i]	AZA[j]	Pred[k]
1	Male	Male	47	0.2	4	0-1-1	350		2000		7.5
2	Male	Female	38	8.1	0	1-1-1				125	10
3	Male	Female	36	2.1	0	0-1-1				175	10
4	Male	Female	62	0.7	0	2-2-2		2	1000		10
5	Female	Female	23	4.3	0	1-0-0				125	10
6	Female	Male	19	0.4	14	1-1-0	200				7.5
7	Male	Female	54	0.7	5	0-2-1	200		2000		7.5
8	Female	Male	43	7.7	0	1-1-1				100	7.5
9	Male	Female	32	1.5	2	1-1-1	200		2000		10
10	Male	Male	61	0.9	2	1-1-1	350		1500		10
11	Female	Male	40	1.1	4	1-1-0	200		1500		
12	Male	Male	48	2.0	9	1-2-2	300				15
		median	41	1.3	2	1-1-1	200	2	1750	125	10

[a]ID, identification number; [b]Pat, patient; [c]Don, donor; [d]KTx, kidney transplantation; [e]PRA, panel-eactive antibodies; [f]HLA-mm, mismatches at the HLA-A, B, and DR loci; [g]CsA, cyclosporine A; [h]Tacro, tacrolimus; [i]MMF, mycophenolate mofetil; [j]AZA, azathioprine; [k]Pred, prednisone.

the cells were lysed with ice-cold milli-Q water and washed extensively. Subsequently, the wells were incubated overnight at 4°C with 100 µl of diluted biotinylated goat anti-human cytokine (IFN-γ, IL-10, IL-13 or GrB) polyclonal antibody (U-CyTech Biosciences) followed by incubation with 50 µl phi-labelled goat anti-biotine antibodies (U-CyTech Biosciences) for 1 h at 37°C. After washing the wells, 30 µl of reagent (activator I + II, U-CyTech Biosciences), that activates phi, was added and incubated for 15 to 30 minutes at room temperature in the dark. The reaction was stopped by adding milli-Q water to the wells. The spots were counted automatically by using a Bioreader 3000 Elispot reader (BioSys, GmbH, Karben, Germany).

mHAg typing after HLA-identical living-related kidney transplantation

PBMC of the patient and the donor were typed for 11 known mHAgs: HA-1, HA-2, HA-3, HA-8, HB-1, ACC-1, ACC-2, HwA-9, HwA-10, UGT2B17 and HY (Table 2). From the 13 HLA-identical donor-recipient couples, 10 couples (ID1, 2, 3, 4, 6, 8, 10, 11, 12, 13) were typed for 11 known mHAgs, and 3 couples (ID5, 7, 9) were not typed for HwA-10. As described, DNA from donor and recipient was isolated using the QIAamp® DNA Mini Kit.[22]

Statistical analysis

The Kruskal-Wallis Test was used to compare age and gender between healthy individuals, and HLA-mismatched and HLA-identical LR kidney transplant recipients. We used the Mann-Whitney *U*-test to compare time after kidney transplantation, serum creatinine, and proteinuria between HLA-mismatched and HLA-identical LR kidney transplant recipients. To compare the frequencies of cytokine pc between healthy individuals, HLA-mismatched and HLA-identical LR kidney transplant recipients the Mann-Whitney *U*-test was used. The Wilcoxon signed rank test was used to compare the number of donor-reactive cytokine pc with the number of their autologous control in PBMC from HLA-identical LR kidney transplant recipients, and the frequencies of IFN-γ pc, IL-10 pc, IL-13 pc and GrB pc within PBMC from healthy individuals, HLA-mismatched LR kidney transplant recipients, and HLA-identical LR kidney transplant recipients. Two sided P-values ≤ 0.05 were considered significant. For statistical analysis, SPSS 11.5 for Windows was used (SPSS, Inc., Chicago, IL, USA).

RESULTS

Clinical results

At time of blood collection, all patients were in a clinical stable period, none of the patients had acute rejection or infections. There was no difference in time after transplantation ($p=0.54$) and panel-reactive antibodies (PRA) ($p=0.60$) between both patient groups (Table 1A and B). Serum creatinine levels were comparable between the HLA-identical group and the HLA-mismatched group ($p=0.54$). None of the HLA-identical and HLA-mismatched recipients had proteinuria (<0.5 g/l). Median age of the HLA-identical and HLA-mismatched recipients and healthy individuals was 49 years (range: 29-58), 41 years (range: 19-62) and 40 years (range: 26-61), respectively. No difference in age ($p=0.82$) and gender ($p=0.61$) was found between kidney transplant recipients and healthy individuals.

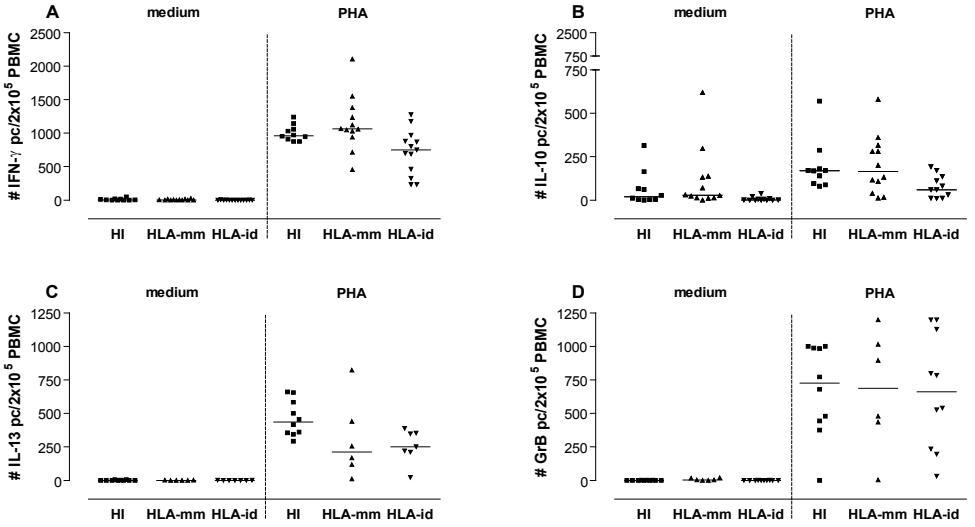

Figure 1: Cytokine responses in culture medium alone and PHA responses in PBMC from healthy individuals (HI), HLA-mismatched (HLA-mm) and HLA-identical (HLA-id) living-related (LR) kidney transplant recipients determined in IFN-γ (A), IL-10 (B), IL-13 (C) and granyzme B (GrB) (D) Elispot assay.

Alloreactivity in PBMC from healthy individuals, HLA-mismatched and HLA-identical LR kidney transplant recipients

The frequency of IFN-γ producing cells (pc) directed to alloantigens in PBMC from healthy individuals (median, 17 IFN-γ pc/2x10^5 PBMC; range, 0-176) was significantly higher than in PBMC from HLA-mismatched (median, 4 IFN-γ pc/2x10^5 PBMC; range, 0-36; p=0.03) and HLA-identical recipients (median, 1 IFN-γ pc/2x10^5 PBMC; range, 0-22; p=0.003; Figure 2A). The number of donor-reactive IFN-γ pc was comparable between the HLA-mismatched and HLA-identical group (p=0.27).

The healthy control group showed low numbers of alloreactive IL-10 pc (median, 0 IL-10 pc/2x10^5 PBMC; range, 0-20), which was comparable to the number of donor-reactive IL-10 pc of the HLA-mismatched group (median, 0 IL-10 pc/2x10^5 PBMC; range, 0-142; p=0.97, Figure 2B). Remarkably, PBMC from the HLA-identical group (median, 13 IL-10 pc/2x10^5 PBMC; range, 0-145) demonstrated higher frequencies of donor-reactive IL-10 pc compared to the number of alloreactive IL-10 pc in PBMC from the healthy control group (p=0.03) and HLA-mismatched group (p=0.04).

Additionally, we analysed whether increased numbers of IL-10 pc were directed to donor antigens or to auto-antigens. Therefore, we compared the number of IL-10 pc of the uncorrected donor-reactive response with the number of IL-10 pc of the autologous response. The donor-reactive IL-10 pc (median, 20 IL-10 pc/2x10^5 PBMC; range, 3-185) was significantly higher than the autologous reactive IL-10 pc (median, 12 IL-10 pc/2x10^5 PBMC; range, 2-40; p=0.03; Figure 2B), suggesting that the donor-reactive response was

directed to donor antigens and not reflecting autoreactivity. Also, in the other Elispot assays, the number of cytokine pc was higher after donor stimulation than in the autologous control (Figure 2A, C, D).

The number of alloreactive IL-13 pc (median, 7 IL-13 pc/2x10^5 PBMC; range 2-22) and GrB pc (median, 8 GrB pc/2x10^5 PBMC; range, 0-20) in PBMC from healthy individuals was higher compared to the number of donor-reactive IL-13 pc and GrB pc in PBMC from HLA-mismatched (IL-13: median, 1 IL-13 pc/2x10^5 PBMC; range, 0-12; p=0.03; GrB: median, 5 GrB pc/2x10^5 PBMC; range, 0-28, p=0.56) and HLA-identical recipients (IL-13: median, 0 IL-13 pc/2x10^5 PBMC; range, 0-2; p<0.001; Figure 2C; GrB: median, 1 GrB pc/2x10^5 PBMC; range, 0-4; p=0.001; Figure 2D). The number of IL-13 pc was comparable between both patient groups (p=0.37), but the number of GrB pc was higher in PBMC from HLA-mismatched group compared to HLA-identical group (p=0.02).

In the healthy control group, allostimulated PBMC demonstrated higher numbers of IFN-γ pc compared to the numbers of IL-10 pc (p=0.01), IL-13 pc (p=0.07) and GrB pc (p=0.03; Figure 2). Also, the number of IL-13 pc was higher than the number of IL-10 pc (p=0.05).

The number of donor-reactive IFN-γ pc, IL-10 pc, IL-13 pc and GrB pc was comparable within the HLA-mismatched group (Figure 2).

It has been suggested that the balance between IFN-γ and IL-10 reflects the immune status of transplant recipients in relation to their donor graft.[15] Therefore, we compared the frequency of allospecific IFN-γ pc with the frequency of IL-10 pc. In PBMC from healthy individuals, high numbers of IFN-γ pc were observed in combination with low numbers of IL-10 pc (p=0.01). No relation was found between the number of donor-reactive IFN-γ pc and IL-10 pc in PBMC from HLA-mismatched recipients (p=0.64). Interestingly, PBMC from HLA-identical recipients showed low numbers of donor-reactive IFN-γ pc in combination with high numbers of donor-reactive IL-10 pc (p=0.01; Figure 3).

Minor histocompatibility antigens typing after HLA-identical LR kidney transplantation

We analysed whether donor-reactive responses (≥ 1 cytokine pc) found after HLA-identical LR kidney transplantation were directed to known mismatched mHAgs between donor and recipient (Table 2). The cytomegalovirus (CMV) and Epstein-Barr virus (EBV) serological status had no influence on donor-reactive IFN-γ, IL-10, IL-13 and GrB responses.

All numbers of IL-13 pc and GrB pc were low, and consequently, no relation could be found between the number of IL-13 pc and GrB pc and the number of mHAg mismatches.

From the 13 donor-recipient combinations, 10 couples (ID1, 2, 3, 4, 6, 8, 9, 10, 11, 12) demonstrated mismatches for known mHAgs. Three couples (ID5, 7, 13) had no mismatches for mHAgs. Those 3 patients had no IFN-γ pc directed to donor antigens, but had significant numbers of IL-10 pc.

Five of the 10 patients had the correct HLA-restriction molecule for donor mHAg presentation to recipient T-cells.[23] One of those patients (ID2) had the highest frequency of IFN-γ pc. Unfortunately, not enough cells were available to perform IL-10 Elispot assay. Two other patients (ID1, ID10) demonstrated high numbers of IL-10 pc in combination with significantly lower numbers of IFN-γ pc. On the other hand, two other patients (ID6, ID11) had detectable numbers of IFN-γ pc in combination with no IL-10 pc.

From the five couples (ID3, 4, 8, 9, 12) without the correct HLA-restriction molecule, four patients (ID3, 4, 9, 12) showed high numbers of IL-10 pc and 1 patient (ID8) had no IL-10 pc, all in combination with low numbers of IFN-γ pc (≤ 2 IFN-γ pc/2x10^5 PBMC).

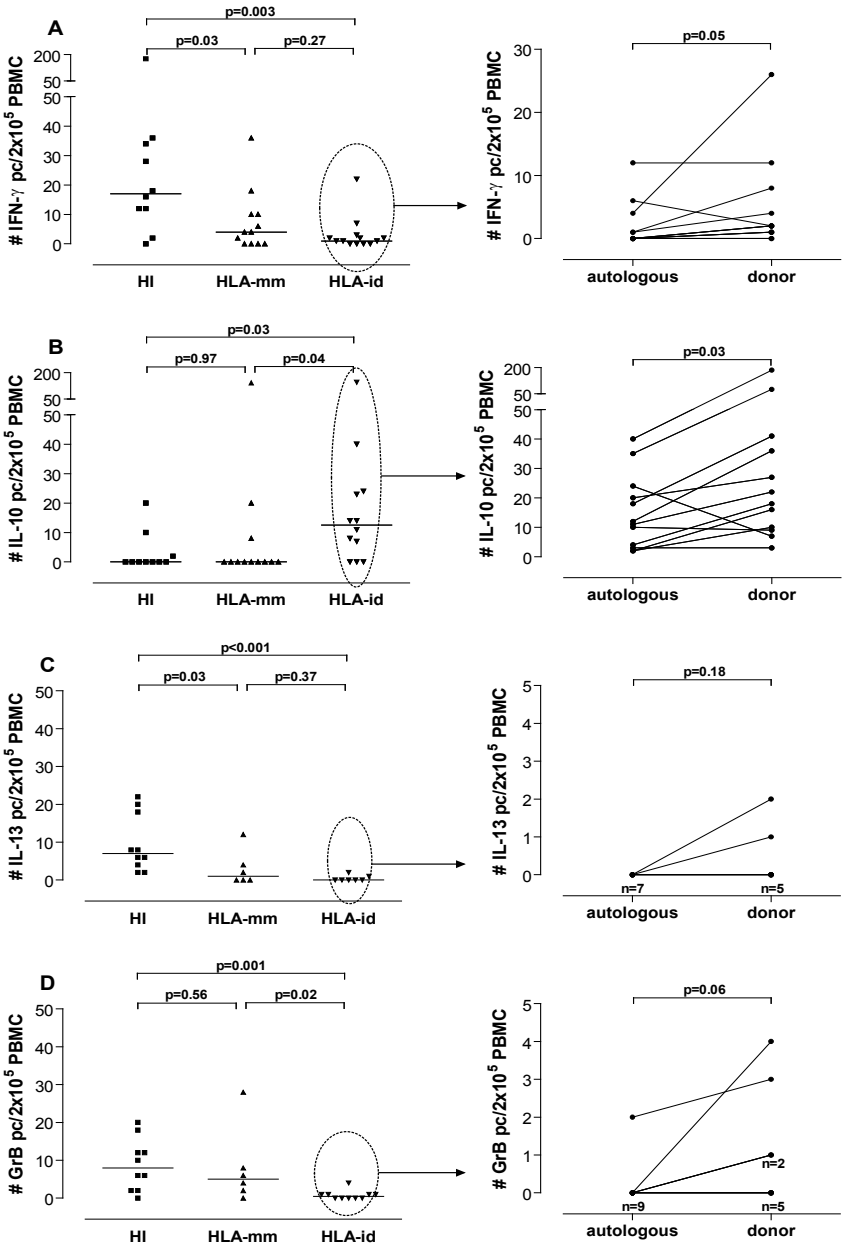

Figure 2: Alloreactivity in PBMC from healthy individuals (HI), and donor-reactive response in patients with an HLA-mismatched (HLA-mm) or HLA-identical (HLA-id) living-related (LR) kidney transplant using IFN-γ (A), IL-10 (B), IL-13 (C), and granzyme B (GrB) (D) Elispot assay. The donor-reactive responses in HLA-identical LR kidney transplant recipients (dotted circles) were further analysed for their autologous responses and uncorrected donor-reactive responses.

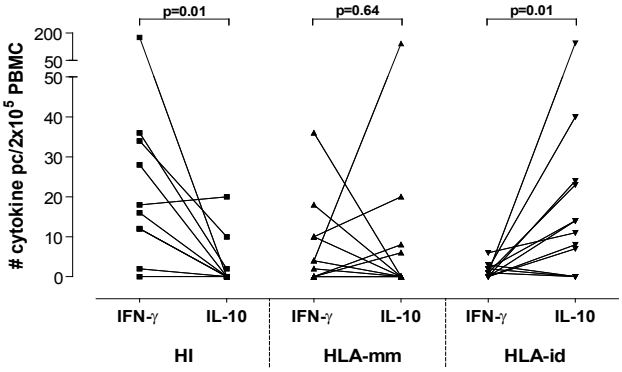

Figure 3: The number of IFN-γ pc and IL-10 pc in PBMC from healthy individuals (HI) after allostimulation. The number of IFN-γ pc and IL-10 pc in PBMC from HLA-mismatched (HLA-mm) and HLA-identical (HLA-id) living-related (LR) kidney transplant recipients after stimulation with donor cells.

Two patients (ID6, ID8) tested more than 3 years after transplantation had no IL-10 pc directed to donor antigens.

DISCUSSION

In humans, mHAg disparities between donor and recipient may influence transplant outcome after BMT.[23] Also, after HLA-identical LR kidney transplantation, theoretically, mismatches in mHAgs between donor and recipient and in combination with the presence of the correct HLA-restriction molecule may trigger T-cell responses.[23] Nowadays, the number of identified human mHAgs have been expanded to a total of 14 autosomally mHAgs and 10 Y-chromosome encoded mHAgs.[22] However, it is yet not known how many mHAgs exist, but is expected to be much more.[3, 24] In our study, during immunological quiescence, we found only in 2 out of 13 patients high donor-reactive IFN-γ pc (>5 IFN-γ pc/2x10^5 PBMC). We observed no donor-reactive IFN-γ pc, IL-13 pc and GrB pc in the absence of known mHAg mismatches. In contrast, high numbers of donor-reactive IL-10 pc were detected in combinations with and without known mHAg mismatches.

Recently, Perez-Garcia *et al.*[25] demonstrated an increased risk for acute GVHD after HA-8 mismatched HLA-identical sibling donor allogeneic BMT, which could cause high T-cell responsiveness directed to donor HA-8. In our study, one couple had an HA-8 mismatch, which could be presented in HLA-A2. This patient had high numbers of donor-directed IFN-γ pc, which suggests a potential risk for graft failure, because HA-8 is also expressed on epithelial cells of the kidney.[26]

IL-10 is a cytokine that has suppressive effects on the production of proinflammatory cytokines and T-cell responses.[27] Several cells produce IL-10, such as activated Th2 cells, monocytes, and T-regulatory type 1 (Tr1) cells.[27, 28] In our experiments, we only used non-adherent cells. Therefore, monocytes do not interfere in the presented Elispot assays. Th2 cells and Tr1 cells can be activated by donor cells.[27] Both Th2 and Tr1 cells mainly produce IL-10. Tr1 cells also produce transforming growth factor (TGF)-β[29], and activated Th2 cells also produce IL-4, IL-5 and IL-13.[28] We found high numbers of donor-reactive IL-10 pc in

Table 2: Known mismatched minor histocompatibility antigens and donor-reactive IFN-γ pc, IL-10 pc, IL-13 pc, and granzyme B pc after HLA-identical LR kidney transplantation.

ID[a]	Gender		Mismatched mHAgs in the HLA-identical donor	HLA-restriction molecule present	Donor-reactive cytokine pc/2x10^5 PBMC			
	Patient	Donor			IFN-γ	IL-10	IL-13	GrB[b]
1	female	male	HA-2M, *HY*[d]	B8, DQ5	1	23	0	4
2	male	female	*HA-8R*, ACC-1Y, ACC-2D, HwA-9G	A2	22	xx[c]	1	1
3	female	female	HA-8R		0	7	xx	xx
4	male	female	HwA-9R		1	145	0	1
5	male	male	No difference		0	8	0	xx
6	female	male	*HY*	B7, B60	1	0	xx	xx
7	male	female	No difference		0	14	0	1
8	male	male	HwA-9G		2	0	0	0
9	male	female	HwA-9G		2	14	2	1
10	female	male	*HB-1H, HY*	B44, DQ5	7	11	xx	0
11	male	male	*HwA-9R*	A3	3	0	xx	0
12	male	female	HA-1R		2	40	xx	0
13	male	female	No difference		0	24	Xx	0

[a]ID, identification number; [b]GrB, Granzyme B; [c]xx, not enough cells to determine 4 cytokines; [d]Mismatched mHAgs with the known HLA-restriction molecule are presented as cursive and underlined.

combination with no or low numbers of IFN-γ pc, IL-13 pc and GrB pc after HLA-identical LR kidney transplantation. Because we did not detect donor-reactive IL-13 pc, we assume that Tr1 cells play an important role in those patients. Unfortunately, we have not determined TGF-β. After BMT with HLA-identical sibling donors, Tr1 cells may play a role via the secretion of IL-10 in preventing GVHD.[13, 14] High frequencies of donor-reactive IL-10 pc determined by Elispot assay were found in PBMC from recipients who did not developed GVHD, while recipients who developed GVHD had low numbers of IL-10 pc.[14] Petersen *et al.*[13] showed high levels of donor-directed IL-10 mRNA in PBMC from recipients who did not developed GVHD, while low IL-10 mRNA levels were found in recipients who developed GVHD. In the HLA-identical LR kidney transplant setting, VanBuskirk *et al.*[30] showed, using a *trans-vivo* delayed-type hypersensitivity (DTH)-analysis, a possible role for Tr1 cells by secretion of IL-10 and TGF-β. Moreover, Rodriguez *et al.*[31] demonstrated, also using a *trans-vivo* DTH-analysis, that matching for HLA molecules resulted in enhanced immune regulation in kidney transplant recipients. In HLA-identical LR kidney transplant recipients, the latter group also found regulated DTH responses, suggesting of the induction of mHAg-specific regulatory T-cell responses. Additionally, Cai *et al.*[32] showed the existence of HA-1-specific T-cells with suppressive function, which was dependent of IL-10, TGF-β and cytotoxic T lymphocyte-associated 4 (CTLA-4), following HLA-identical LR kidney transplantation. In our study, this could suggest that HLA-identical LR kidney transplant recipients are developing peripheral tolerance against non-HLA antigens by Tr1 cells through the secretion of IL-10. Therefore, we propose that HLA-identical LR kidney transplant recipients with stable renal function and high numbers of donor-reactive IL-10 pc in combination with low numbers of IFN-γ pc should be tapered in their immunosuppressive load.

It has been suggested that the presence of PRA reactivity was associated with long-term graft loss in kidney transplants from HLA-identical sibling donors[4], which could reflect immune reactivity directed to non-HLA antigens or mHAgs. In the present study, we found

no correlation between PRA before HLA-identical LR kidney transplantation and the number of IFN-γ pc, IL-10 pc and GrB pc. Also, in the HLA-mismatched group, no relation was found between PRA before kidney transplantation and the number of cytokine producing cells.

After HLA-mismatched kidney transplantation, high donor-reactive IL-10 pc was associated with stable graft function, and low IL-10 was related with rejection.[15] In our study, 3 HLA-mismatched LR kidney transplant recipients with excellent renal function had high numbers of IL-10 pc in combination with low numbers of IFN-γ pc. Because we assume that donor-reactive IL-10 pc could be derived from Tr1 cells, we suggest to reduce the immunosuppressive load in patients with stable graft function and high numbers of donor-reactive IL-10 pc in combination with low numbers of IFN-γ pc. Whether the immunosuppressive medication influences the balance between IFN-γ and IL-10 should be investigated by e.g. *in vitro* addition of immunosuppressive drugs, or by discontinuation the immunosuppressive medication in these transplant recipients. Furthermore, the autologous response was comparable between healthy individuals and transplant recipients (data not shown), suggesting that immunosuppression has no influence on the autologous response.

Studies reported that $CD4^+CD25^{bright}$ regulatory T cells might play a beneficial role in mediating peripheral tolerance after kidney transplantation.[33, 34] Unfortunately, not enough cells were available to determine the percentage $CD4^+CD25^{bright}$ regulatory T cells by flow cytometry.

In conclusion, high numbers of donor-reactive IL-10 pc in combination with low reactivity in autologous control, low numbers of donor-reactive IFN-γ pc, IL-13 pc and GrB pc, after HLA-identical LR kidney transplantation, may reflect active downregulation of reactivity against non-HLA molecules.

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Chapter 5

Donor-reactive cytokine production after HLA-identical living-related kidney transplantation: a protein-array analysis

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ABSTRACT

Background: In the present pilot study, we investigated which proteins are produced after donor stimulation of peripheral blood mononuclear cells from recipients of HLA-identical living-related kidney transplant.

Methods: We used a protein-array analysis to determine cytokines, chemokines, and growth factors in supernatant from donor-stimulated mixed lymphocyte reaction cultures. Autologous cultures were considered to be negative control.

Results: In 38 out of 42 proteins (90%), the donor response was higher compared with the autologous response.

Conclusion: Therefore, we concluded that even after HLA-identical LR kidney transplantation we could measure a donor-reactive response, which we assumed was directed toward minor histocompatibility antigens or to non-HLA antigens.

INTRODUCTION

In HLA-identical living-related (LR) kidney transplantation, all major HLA molecules of the recipient are identical to those of the donor. Nevertheless, minor histocompatibility antigen (mHAg) mismatches may exist. mHAgs are genetically inherited peptides derived from polymorphic intracellular proteins presented in the context of HLA molecules, which can be recognized by the immune system.[1] The expression of mHAgs is ubiquitous or specific on haematopoietic cells.[2] Beside mHAg mismatches, differences in other non-HLA antigens may exist between donor and recipient.[3]

A key problem after HLA-identical LR kidney transplantation is that no donor-reactive responses can be measured by methods as mixed lymphocyte reaction (MLR), cytotoxic T lymphocyte (CTL) precursor frequency, or helper T lymphocyte precursor frequency assays. Recently, we demonstrated that after HLA-identical LR kidney transplantation, responses against donor antigens may be measured by a sensitive method, interferon (IFN)-γ[4] (or granzyme B[5]) Elispot assay. We questioned whether other donor-reactive cytokines can be produced in the HLA-identical LR kidney transplantation setting. Recently, a protein-array system became available to determine protein expression levels of cytokines, chemokines, and growth factors in cell culture supernatants. In this array it is possible to detect multiple cytokine expression levels simultaneously. In the present pilot study, we screened the expression profiles of 42 cytokines in donor-stimulated MLR cultures from peripheral blood mononuclear cells (PBMC) of HLA-identical LR kidney transplant recipients.

MATERIALS AND METHODS

Patients and PBMC sampling

Two stable HLA-identical LR kidney transplant recipients (without proteinuria) participated in this study. Both recipients suffered from membranous glomerulonephritis before transplantation. Recipient 1 was 2.1 years after kidney transplantation and had received mycophenolate mofetil (1000 mg/day) in combination with tacrolimus (4 mg/day). Recipient 2 was 0.5 year after kidney transplantation and received cyclosporine A (350 mg/day) and prednisone (10 mg/day). PBMC were isolated from heparinized blood by density gradient centrifugation using Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) as described before.[4] Thereafter, PBMC were stored in RPMI-1640-DM (Cambrex, Verviers, Belgium) containing 15% foetal calf serum and 10% dimethyl sulfoxide (MERCK, Germany) at –140 ^{o}C.

MLR

Into a 96-well round bottom plate (Nunc, Roskilde, Denmark), we added 100 µl of 5 x 10^4 patient PBMC in complete culture medium [RPMI-1640-DM (Cambrex) supplemented with 2 mmol/l of L-glutamine (GibcoBRL, Scotland, United Kingdom), 100 IU/ml of penicillin (Cambrex), 100 µg/ml of streptomycin (Cambrex), and 10% heat-inactivated and filtered (0.20-µm sterile syringe filter, Corning Incorporated, Corning, NY) human male serum that was pre-tested for adequate cell growth support in MLR] to 100 µl of 5 x 10^4 irradiated (40 Gy) donor PBMC. To control the influence of irradiation on cytokine production, 5 x 10^4 patient PBMC were incubated with 5 x 10^4 irradiated (40 Gy) patient PBMC (autologous

response). After incubation of 7 days at 37 °C and 5% CO_2, 200 µl cell culture supernatant was obtained and stored at −20 °C until use.

Human Cytokine Antibody Array

RayBio® Human Cytokine Antibody Array III (RayBiotech Inc, Norcross, GA, USA) was used to assay cell culture supernatants from the above mentioned MLR to determine 42 proteins, including cytokines, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40p70, IL-13, IL-15, IFN-γ, TGF-β1, TNF-α, TNF-β; chemokines, GRO, CXCL1 (GRO-α), CXCL5 (ENA-78), CXCL9 (MIG), CXCL12 (SDF-1), CCL1 (I-309), CCL2 (MCP-1), CCL5 (RANTES), CCL7 (MCP-3), CCL8 (MCP-2), CCL15 (MIP-1δ), CCL17 (TARC), CCL22 (MDC); and growth factors, GCSF, GM-CSF, MCSF, SCF, EGF, IGF-1, Angiogenin, oncostatin M, Thrombopoietin, VEGF, PDGF-BB, Leptin. As positive controls a biotin-conjugated IgG was provided by the manufacturer. The intensity of this positive signal (100%) was used to determine the relative protein expression levels of all samples measured. Small changes in the manufacturer's recommended protocol were made: the membrane was treated for 6 hours with blocking buffer instead of 30 minutes, and the samples were diluted 1:10 in blocking buffer. To determine the relative protein expression levels, we used a MultiImage® Light Cabinet (Alpha Innotech Corporation, San Leandro, California, USA).

RESULTS

Inflammatory proteins are a group of proteins that act as mediators of cell communication. Based on this definition, inflammatory proteins include cytokines, chemokines, and growth factors. Therefore, we presented our data in three groups: cytokines, chemokines, and growth factors. The results are expressed as relative expression compared to a positive control included in the membrane of the array. The relative increase in cytokines (Figure 1, 1A and 2A), chemokines (Figure 1, 1B and 2B), and growth factors (Figure 1, 1C and 2C) was determined in culture supernatants from donor-stimulated MLRs compared to autologous control supernatants.

The donor response was higher than the autologous response for 15 of 17 (88%) cytokines in supernatant from recipient 1 (Figure 1, 1A), and 14 out of 17 (82%) cytokines in recipient 2's supernatant (Figure 1, 2A). We also observed a greater donor than autologous response for 13 out of 13 (100%) chemokines in supernatant from recipient 1 (Figure 1, 1B), and 12 out of 13 (92%) chemokines in supernatant from recipient 2 (Figure 1, 2B). In addition, for 10 out of 12 (83%) growth factors determined in a supernatant from recipient 1 (Figure 1, 1C) and 12 out of 12 (100%) growth factors in the supernatant from recipient 2, the donor response was higher than the autologous response (Figure 1, 2C).

DISCUSSION

Advantages of the protein-array method include high sensitivity, simultaneous detection of multiple protein expression levels simultaneously, and easy performance. The array is a screening method. Thereafter, the exact concentration of proteins or cytokine producing cells of interest should be determined in ELISA or Elispot, respectively.

Recently, we demonstrated frequencies of IFN-γ and granzyme B producing cells

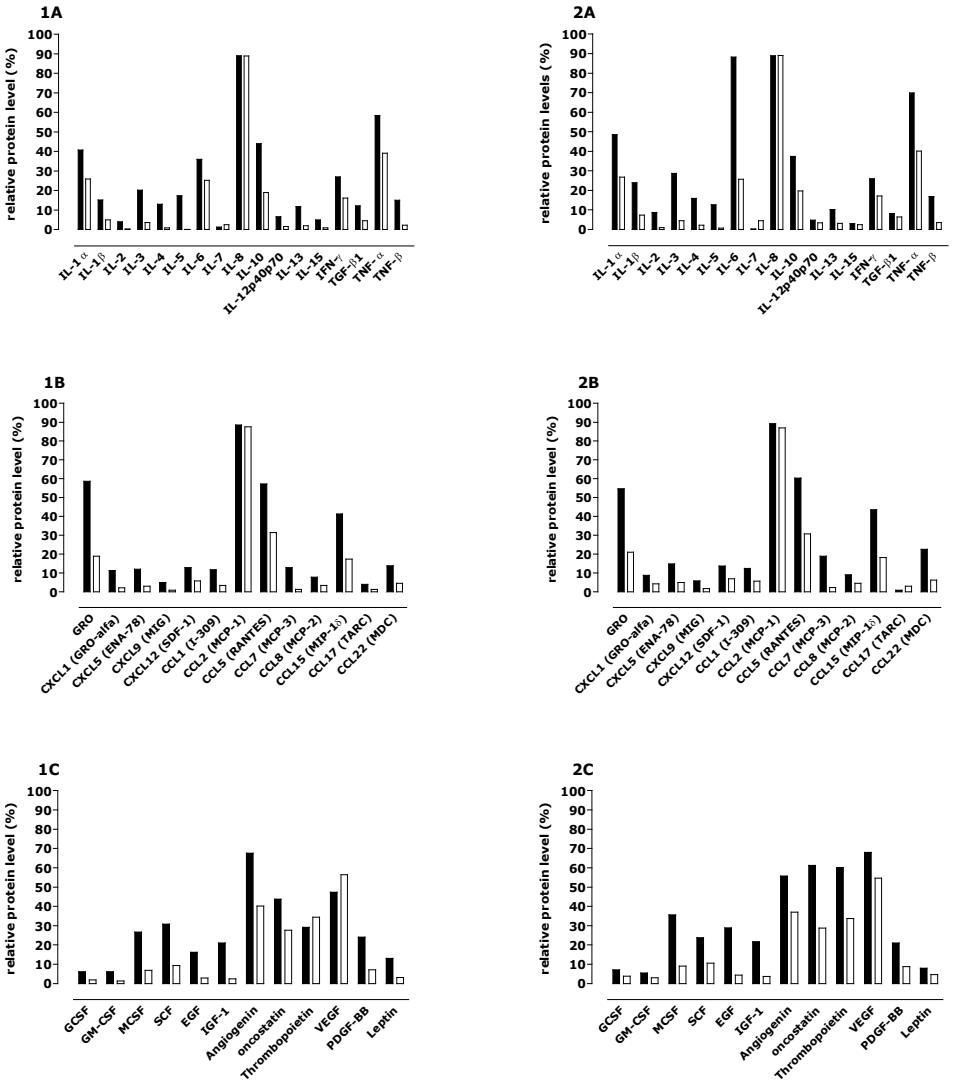

Figure 1: Relative expression profile of cytokines (1A: recipient 1, 2A: recipient 2), chemokines (1B: recipient 1, 2B: recipient 2), and growth factors (1C: recipient 1, 2C: recipient 2) determined in donor-stimulated MLR cultures from PBMC of HLA-identical LR kidney transplant recipients (black bars). Autologous stimulated cultures were considered as negative control (white bars). The intensity of a positive control (100%) was used to determine the relative expression levels of the inflammatory proteins.

specific for donor antigens as determined by Elispot assay in recipients of HLA-identical LR kidney transplantation.[4, 5] In addition, we found detectable numbers of IL-10 and IL-13 producing cells in PBMC from some recipients of an HLA-identical LR kidney transplant, suggesting a T-helper 2-skewed alloreactivity directed to donor mHAgs or to non-HLA antigens.[6] In the present study, we demonstrate that donor-reactive cytokine responses can even be observed for 90% (38/42) of the cytokines determined by a protein-array method. These results confirm our earlier results of donor-reactive cytokine producing cells directed toward donor antigens as detected by Elispot after HLA-identical LR kidney transplantation.[4, 5] We assume that the donor responses are directed toward donor mHAgs or toward non-HLA antigens.

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Chapter 6

Peripheral blood manipulation significantly affects the result of dendritic cell monitoring

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ABSTRACT

Background: It has been postulated that the plasmacytoid/myeloid dendritic cell ratio (pDC/mDC) reflects immune reactivity, and can therefore be used to monitor transplant recipients. We investigated the influence of Ficoll-Paque separation and PBMC cryopreservation on the pDC/mDC ratio and the expression of maturation markers, e.g. chemokine receptors (CKR) CCR7, CXCR4, and CCR5, in comparison to fresh blood cells.

Methods: Fractions of pDC and mDC, and CKR expression were measured by flow cytometry in fresh blood, in Ficoll-isolated PBMC and in cryopreserved PBMC from healthy individuals and kidney transplant recipients.

Results: Ficoll-isolation of PBMC resulted in higher pDC/mDC ratios in both groups compared to fresh blood cells resulting from a relatively large increase in pDC compared to mDC. The pDC/mDC ratio increased further after cryopreservation of PBMC from kidney transplant recipients. Ficoll-isolation and cryopreservation of PBMC affected the proportion of mDC and pDC positive for CKR, and their expression levels resulting in a more mature phenotype.

Conclusions: The pDC/mDC ratio and pDC or mDC maturation status based on CKR expression, is dependent on manipulation of PBMC. Therefore, fresh blood is preferable for monitoring purposes in transplant patients, as only these cells reflect the in vivo immune-status of patients accurately.

INTRODUCTION

Dendritic cells (DC) are professional antigen presenting cells that play a critical role in the induction and regulation of immune responses. During their lifetime, DC are not only found in lymphoid tissues, but also in blood and in non-lymphoid tissues, where they act as sentinels for antigen capture. DC have different functions at various stages of maturation. Immature DC capture antigens by endocytosis, phagocytosis and pinocytosis, and differentiate into mature DC.[1] Immature DC express chemokine receptors (CKR) CCR1, CCR2, CCR5, CXCR3 and CXCR4, low levels of MHC class I and II molecules, and do not express CD83, CD54, CD80/CD86.[2-6] Mature DC are highly specialised to activate T cells in the lymphoid organs and are characterised by high expression of CD83, CD54, CD80/86, and MHC class I and II molecules. Besides the upregulation of costimulatory molecules, the expression of CCR7 is enhanced, whereas CCR5 and CXCR4 is downregulated compared to immature DC.[5, 7-10]

In human peripheral blood, at least two DC subsets have been described: the $CD11c^{+}CD123^{low}$ myeloid DC (mDC) and the $CD11c^{-}CD123^{high}$ plasmacytoid DC (pDC).[11] Whereas mDC produce large amounts of IL-12 and induce strong T-helper 1 and cytotoxic T lymphocyte (CTL) responses, pDC induce T-helper 2 responses and produce lower amounts of IL-12. Additionally, pDC express the IL-3 receptor chain α (CD123), which is necessary for survival and differentiation, and are the main producers of IFN-α in response to viruses.[12]

The absolute number of pDC and mDC or their ratio (pDC/mDC) in peripheral blood could be useful for immunological monitoring in disease and organ transplantation.[13-18] Several studies have shown that the numbers of mDC and pDC in peripheral blood vary according to conditions such as exercise, stress, surgery, cancer, and leukemia, and lower mDC and pDC numbers have been reported in acquired immunodeficiency syndrome.[13-16, 18-20] In patients with acute myeloid leukemia, higher percentages of both pDC and mDC were observed in comparison to healthy individuals.[15] In contrast, mDC were higher during end stage heart failure and had a partially mature phenotype based on their CCR7 and CD83 expression.[21] After clinical heart transplantation, the absolute number of pDC and mDC in peripheral blood is strongly reduced[21], and the number of mDC decreased further during acute rejection.[20] Mazariegos *et al.*[17] showed that the ratio between pDC and mDC in peripheral blood of stable liver transplant patients was higher in patients who could be successfully weaned from immunosuppression.

Most of the aforementioned studies used a cocktail of DC Lineage (Lin1 method) negative monoclonal antibodies (mAbs), which includes mAbs for T cells, B cells, NK cells, and monocytes, in combination with HLA-DR, CD123 and CD11c mAbs.[14, 15, 17, 18, 20, 21] Upham *et al.*[13] used CD33 mAbs for identifying mDC, and Ho *et al.*[19] used CMRF-44 mAbs for identifying peripheral blood DC. Recently, a new set of mAbs became available for identifying mDC and pDC: the blood dendritic cell antigen (BDCA)-1 mAbs are used for mDC, and BDCA-3 mAbs for a very small fraction of mDC which lack the expression of BDCA-1. BDCA-2 mAbs and BDCA-4 mAbs are used for identifying pDC.[22] With the BDCA antibodies, it became possible to determine pDC and mDC without the use of a large number of Lineage-negative markers and anti-HLA-DR. Therefore, determination of the maturation status of both DC subsets is easier and more efficient compared to the Lin1-method.

In the described studies, variable cell-preparations were used. Most authors used PBMC isolated by Ficoll-Paque separation, with or without subsequent cryopreservation.[14, 15, 17, 19] Only Upham *et al.*[13] and Athanassopoulos *et al.*[20, 21] used fresh blood samples. However, in PBMC samples it is impossible to determine the absolute number of DC/µl blood. In addition, it is also unknown whether Ficoll-Paque separation alters the ratio between pDC

and mDC, or affects the maturation status of both DC subsets.

Objective

Since, it is practical and probably more accurate to measure sequential blood samples of one patient in one flow cytometric session, we investigated the influence of PBMC cryopreservation and storage on the pDC/mDC ratio and maturation status of DC subsets. Therefore, we compared the pDC/mDC ratio and the maturation status of the DC subsets in fresh blood, and in the same sample after Ficoll-Paque separation or subsequent cryopreservation. All tests were performed with the BDCA antibodies. In addition, we assessed the reproducibility of the BDCA method. Finally, in order to control the effect of immunosuppression, DC subsets were measured in fresh blood cells and cryopreserved PBMC from heart failure patients, who did not receive immunosuppression, by using the Lin1 method.

MATERIALS AND METHODS

Study group

The study population consisted of healthy individuals (n=10), kidney transplant (KTx) recipients (n=9), and heart failure (HF) patients (n=5). Informed consent was obtained from each individual. Seven of the KTx recipients received a living-related donor kidney (KTx 1, 3, 4, 5, 6, 7, 9), and 2 recipients received a cadaveric donor kidney (KTx 2, 8). All KTx patients were selected at least 2 years after kidney transplantation (median, 5.9 years; range, 2.7-9.4). Median age of the healthy individuals, KTx recipients and HF patients was 31.4 years (range, 24.4-55.6), 40.8 years (range, 26.4-51.3) and 57.8 years (range, 39.2-62.4), respectively. Healthy individuals were significantly younger than KTx ($p=0.002$) and HF patients ($p=0.003$). No difference in age was found between KTx recipients and HF patients ($p=0.90$). Healthy individual and patient characteristics are described in Table 1.

Peripheral Blood Mononuclear Cells Sampling

From healthy individuals, KTx recipients and HF patients, 35 ml heparinized blood was obtained. 2 ml was used for fresh blood measurements, and 33 ml was used for isolation of peripheral blood mononuclear cells (PBMC) and subsequent cryopreservation. All blood samples were processed and measured within 1-3 h after collection.

PBMC were isolated from heparinized blood by density gradient centrifugation using Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden). PBMC were collected from the interphase, washed twice with RPMI-1640-DM (GibcoBRL, Scotland, United Kingdom) supplemented with 100 IU/ml of penicillin (Cambrex, Verviers, Belgium) and 100 µg/ml of streptomycin (Cambrex). To determine the ratio pDC/mDC, approximately $10x10^6$ PBMC were used for direct flow cytometry.

Approximately $2x10^7$ PBMC (PBMC-CRYO) were frozen in 2 ml RPMI-1640-DM containing 15% foetal calf serum (FCS) and 10% dimethyl sulfoxide (MERCK, Darmstadt, Germany) and stored at −80°C for at least 7 days. For successful cryopreservation of PBMC, Nalgene® Cryo Freezing containers (Nalgene Nunc International, Rochester, New York) were used, achieving a −1°C/minute rate of cooling when placed at −80°C. After thawing of the PBMC, cell viability was >95%.

Table 1: Healthy individual and patient characteristics

		Sex[1] M/F	Age (years)	Time after Tx[9] (years)	Immunosuppression (per day)
HI[2]	1	M	26.0		-
	2	F	52.2		-
	3	M	28.1		-
	4	M	30.5		-
	5	F	24.4		-
	6	F	35.7		-
	7	M	55.6		-
	8	F	32.3		-
	9	M	25.7		-
	10	F	38.8		-
KTx[3]	1	M	38.6	9.4	150 mg AZA[5] + 10 mg pred
	2	M	45.4	7.9	2 mg Tacro[6] + 1000 mg MMF[7] + 2.5 mg pred
	3	M	40.8	5.3	50 mg AZA + 5 mg pred[8]
	4	M	45.1	5.9	50 mg AZA + 10 mg pred
	5	M	30.8	6.1	75 mg AZA + 7.5 mg pred
	6	M	51.3	6.7	1500 mg MMF + 5 mg pred
	7	M	29.3	4.3	100 mg AZA + 10 mg pred
	8	F	26.4	2.7	4 mg Tacro + 5 mg pred
	9	F	50.2	3.9	8 mg Tacro + 500 mg MMF
HF[4]	1	M	62.4		Statines, Becotide
	2	F	39.2		-
	3	F	57.8		-
	4	M	59.0		Statines
	5	M	56.3		Pulmicort

[1]M, male; F, female; [2]HI, healthy individuals; [3]KTx, kidney transplant recipients; [4]HF, heart failure patients; [5]AZA, azathioprine; [6]Tacro, tacrolimus; [7]MMF, mycophenolate mofetil; [8]pred, prednisone; [9]Tx, transplantation.

Antibodies for staining

For the BDCA method the following mouse anti-human monoclonal antibodies (mAbs) were used: allophycocyanin (APC)-conjugated BDCA-1 (clone: AD5-8E7) and APC-conjugated BDCA-2 (clone: AC144) (both purchased from Miltenyi Biotec, GmbH, Germany), peridinin chlorophyll protein (PerCP)-conjugated CD14 (clone: mφP9) and PerCP-conjugated CD19 (clone: 4G7; both purchased from Becton Dickinson Biosciences, San Jose, CA, USA), fluorescein isothiocyanate (FITC)-conjugated CCR5 (CD195; clone: 2d7) and phycoerythrin (PE)-conjugated CXCR4 (CD184; clone: 12G5; both purchased from Becton Dickinson Pharmingen, San Diego, CA, USA), PE-conjugated CCR7 (clone: 150503; purchased from RD Systems, Minneapolis, USA). For the Lin1 method the following mouse anti-human mAbs were used: FITC-conjugated CD34 (clone 8G12), FITC-conjugated Lineage cocktail 1 (containing CD3 (clone: SK7), CD14 (clone: MφP9), CD16 (clone: 3G8), CD19 (clone: SJ25C1), CD20 (clone: L27), and CD56 (clone: NCAM16.2)), PE-conjugated anti-IL-3 receptor α chain (CD123) (9F5), APC-conjugated CD11c (clone: S-HCL.3), PerCP-conjugated anti-HLA-DR (clone: L243) (all purchased from Becton-Dickinson Biosciences, San Jose, CA, USA).

Immunofluorescence staining and flow cytometric analysis – BDCA method

Analysis of mDC and pDC was performed by four-colour flow cytometry. BDCA-1 and BDCA-2 were used to identify mDC and pDC, respectively. Another marker for identifying pDC is BDCA-4. However, staining with BDCA-2 mAbs results in a more compact cluster of cells

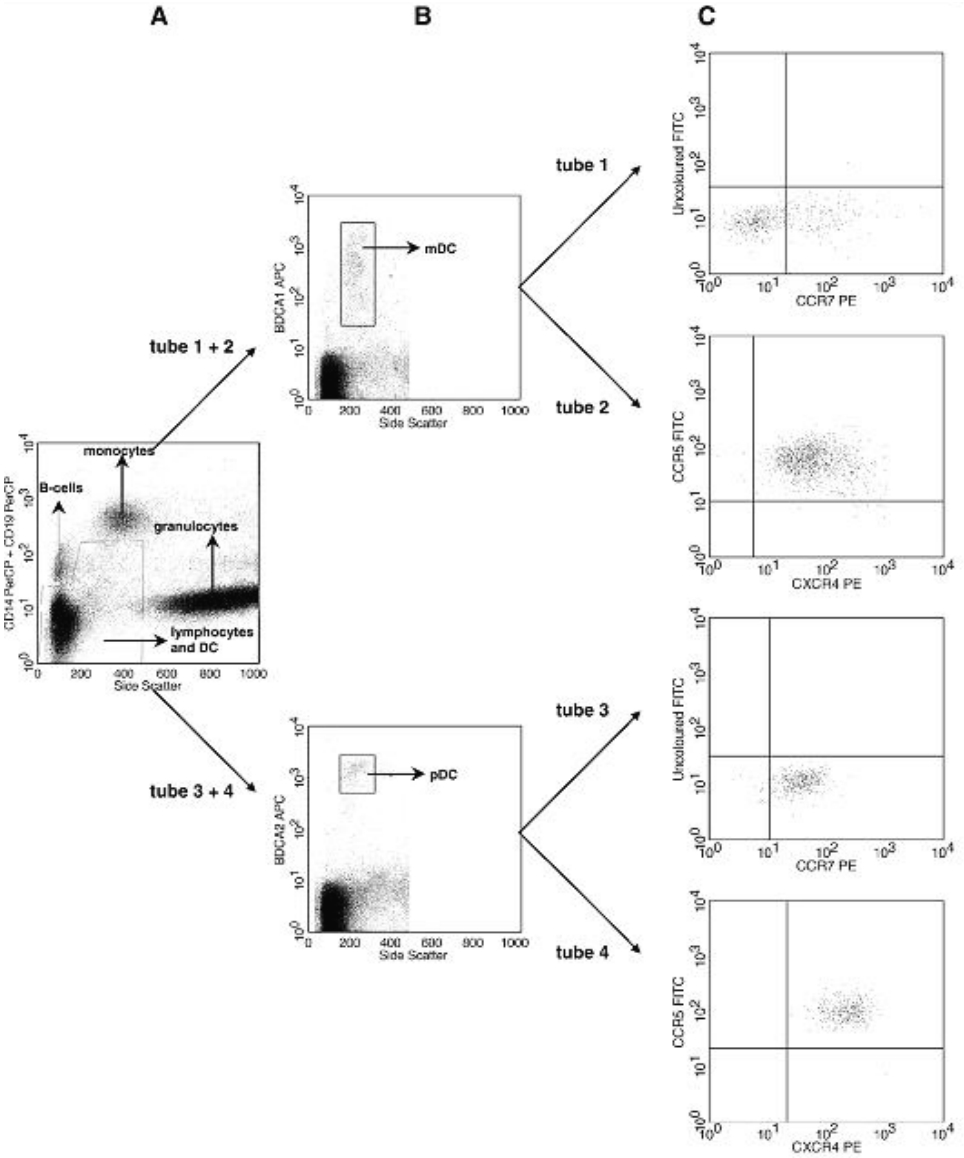

Figure 1: Identification of peripheral blood dendritic cells (DC) phenotype (in healthy individuals and kidney transplant recipients). DC were identified in CD14 negative and CD19 negative population (A). Myeloid DC (mDC) and plasmacytoid DC (pDC) were further analysed by high expression of BDCA-1 and BDCA-2, respectively (B). Characterization of the maturation status for both DC subsets was achieved by analysis of the chemokine receptor markers CCR7, CCR5 and CXCR4 (C).

than BDCA-4 mAbs [22], thus only BDCA-2 mAbs was used in this study.
In tubes 1 and 2, 300 µl fresh blood cells were incubated with 10 µl BDCA-1-APC mAbs, 15 µl CD14-PerCP mAbs, and 30 µl CD19-PerCP mAbs. In tubes 3 and 4, 300 µl fresh blood cells were incubated with 10 µl BDCA-2-APC mAbs, 15 µl CD14-PerCP mAbs, and 30 µl CD19-PerCP mAbs. In tubes 1 and 3 also 15 µl CCR7-PE mAbs were added, whereas in tubes 2 and 4, 30 µl CCR5-FITC mAbs and 30 µl CXCR4-PE mAbs were added. Cells were incubated for 30 min in the dark at room temperature. Thereafter, erythrocytes were lysed with 2 ml FACS lysing solution (Becton Dickinson) for 10 minutes in the dark at room temperature. Subsequently, cells were centrifuged for 5 min at 2000 rpm and supernatant was discarded. Cells were washed twice in 2 ml FACSflow (Becton Dickinson) and centrifuged for 5 min at 2000 rpm. Thereafter, cells were resuspended in at least 300 µl FACSflow.

Ten million Ficoll-isolated PBMC were washed with 2 ml FACSflow and centrifuged for 5 min at 2000 rpm. Thereafter, PBMC were resuspended in 1.5 ml FASCflow. Subsequently, 300 µl cell suspension was added to the mAb cocktails as described for fresh blood cells, and incubated for 30 min in the dark at room temperature. After incubation, cells were washed twice with FACSflow, and at least 300 µl FACSflow was added to PBMC.

After cryopreservation, PBMC were thawed, and $10x10^6$ cells were used to determine the pDC and mDC fractions. First, PBMC were washed in 2 ml FACSflow and resuspended in 1.5 ml FASCflow. 300 µl cell suspension was added to the mAb cocktails as described for fresh blood cells, and incubated for 30 min in the dark at room temperature. After incubation, cells were washed twice with FACSflow and resuspended in 300 µl FACSflow.

From each tube, 500 000 events were acquired on a FACScalibur flow cytometer using CELLQUEST PRO software (Becton Dickinson). As illustrated in Figure 1, $CD14^-$ and $CD19^-$ gated cells were analysed for BDCA-1 or BDCA-2 expression (Figure 1A). mDC and pDC were identified as $CD14^-CD19^-BDCA\text{-}1^+$ cells and $CD14^-CD19^-BDCA\text{-}2^+$ cells, respectively (Figure 1B). In 6 healthy individuals and 4 KTx patients, the proportion of mDC and pDC positive for CCR7, CCR5 and CXCR4 (Figure 1C) and their geometric Mean Fluorescence Intensity (gMFI) was determined.

Immunofluorescence staining and flow cytometric analysis – Lineage 1 method

Analysis of mDC and pDC with the Lin1 method was performed as described recently by Athanassopoulos.[21] Briefly, 200 µl of fresh blood samples were incubated with 5 µl Lin1-FITC and 5 µl CD34-FITC mAbs, 5 µl HLA-DR-PerCP mAbs, 10 µl CD11c-APC mAbs (1:20 diluted in FACSflow), and 10 µl CD123-PE mAbs (1:5 diluted in FACSflow) for 30 min in the dark at room temperature. Subsequently, erythrocytes were lysed with 2 ml FACS lysing solution for 10 min in the dark at room temperature. Next, cells were centrifuged for 5 min at 2000 rpm, and supernatant was discarded. Cells were washed twice with 2 ml FACSflow, and resuspended in 300 µl FACSflow.

Cryopreserved PBMC were thawed, washed in 2 ml FACSflow and resuspended in 1.5 ml FASCflow at a concentration of $5x10^6$ PBMC/ml. 300 µl cell suspension was added to the same amount of mAbs as described for fresh blood cells, and incubated for 30 min in the dark at room temperature. After incubation, cells were washed twice with FACSflow, and resuspended in 300 µl FACSflow.

From each tube, 300 000 events were acquired on a FACScalibur flow cytometer using CELLQUEST PRO software. Cells that stained negative for Lin1-cocktail and positive for HLA-DR expression were gated, and further defined for the expression of CD11c and CD123, which represents mDC and pDC, respectively.

Biovariability and intra-assay reproducibility of the BDCA method

Biovariability of the BDCA method was performed in 5 healthy individuals (C1, C7, C8, C9, C10; Table 2). 7 ml of fresh blood cells from each individual was collected and the mDC and pDC fractions were measured at week 1, week 2, and week 3.

Intra-assay reproducibility of the BDCA method was performed in 4 healthy individuals (C2, C7, C8, C9; Table 3). The staining as described in Section 'Immunofluorescence staining and flow cytometric analysis - BDCA method' was performed 5 times separately using the same blood sample to measure the pDC and mDC fractions.

Statistical analysis

The Wilcoxon signed rank test was used to compare the pDC/mDC ratio between fresh blood samples, PBMC, and PBMC-CRYO in healthy individuals, KTx recipients, and HF patients. The Wilcoxon signed rank test was also used to compare the percentage positive and expression levels of CCR7, CCR5, and CXCR4 on DC subsets between fresh blood samples, PBMC, and PBMC-CRYO in healthy individuals and KTx recipients. The Mann-Whitney U-test was used to compare the age of healthy individuals, KTx recipients and HF patients. Two sided p-values ≤ 0.05 were considered significant. The coefficient of variation (CV) was used to measure biovariability and intra-assay reproducibility. For statistical analysis, SPSS 11.5 for Windows was used (SPSS, Inc., Chicago, IL, USA).

Table 2: Biovariability of the BDCA method

		BDCA method		
Individual	**Week**	**% pDC**	**% mDC**	**pDC/mDC ratio**
1	1	28	72	0.38
	2	33	67	0.50
	3	37	63	0.58
	Mean	32.7	67.3	0.49
	SD	4.5	4.5	0.10
	CV %	13.8	6.7	
2	1	31	69	0.45
	2	26	74	0.35
	3	26	74	0.35
	Mean	27.7	72.3	0.38
	SD	2.9	2.9	0.06
	CV %	10.4	4.0	
3	1	46	54	0.86
	2	32	68	0.46
	3	40	60	0.67
	Mean	39.3	60.7	0.67
	SD	7.0	4.0	0.20
	CV %	17.9	11.6	
4	1	38	62	0.61
	2	40	60	0.68
	3	38	62	0.62
	Mean	38.7	61.3	0.64
	SD	1.2	1.2	0.04
	CV %	3.0	1.9	
5	1	24	76	0.31
	2	20	80	0.24
	3	17	83	0.21
	Mean	20.3	79.7	0.25
	SD	3.5	3.5	0.05
	CV %	17.3	4.4	

Table 3: Intra-assay reproducibility of the BDCA method

		BDCA method		
Individual	**measurement**	**%pDC**	**%mDC**	**pDC/mDC ratio**
1	1	26	74	0.35
	2	29	71	0.40
	3	31	69	0.44
	4	29	71	0.40
	5	26	74	0.34
	Mean	28.2	71.8	0.39
	SD	2.2	2.2	0.04
	CV %	7.7	3.0	
2	1	20	80	0.25
	2	29	71	0.41
	3	26	74	0.35
	4	21	79	0.27
	5	20	80	0.25
	Mean	24.4	75.6	0.32
	SD	3.2	3.2	0.06
	CV %	13.1	4.2	
3	1	14	86	0.16
	2	11	89	0.13
	3	14	86	0.16
	4	12	88	0.14
	5	14	86	0.17
	Mean	13.0	87.0	0.15
	SD	1.4	1.4	0.02
	CV %	10.9	1.6	
4	1	45	55	0.83
	2	42	58	0.72
	3	45	55	0.81
	4	47	53	0.88
	5	37	63	0.58
	Mean	43.2	56.8	0.77
	SD	3.9	3.9	0.12
	CV %	9.0	6.8	

RESULTS

Biovariability and intra-assay reproducibility of the BDCA method

Blood from healthy individuals were taken on 3 consecutive weeks (Table 2). Within 3 weeks less than 18% variation (CV: range, 3.0-17.9%) existed between the percentage of pDC, and less than 12% variation for the mDC (CV: range, 1.9-11.6%).

Table 3 shows the intra-assay reproducibility of the BDCA method. In all controls, the CV for the pDC (range, 7.7-13.1%) and mDC (range, 1.6-6.8%) fractions was at all times lower than 14%.

Influence of Ficoll-Paque separation and subsequent cryopreservation on dendritic cell subset ratio in healthy individuals and kidney transplant recipients

Proportion of mDC and pDC was determined by flow cytometry from all healthy individuals and KTx recipients.

After Ficoll-isolation (PBMC), the pDC/mDC ratio was higher (median, 0.66; range, 0.36-1.1) than in corresponding fresh blood samples (median 0.54; range, 0.17-0.86; p=0.02; Figure 2A) from healthy individuals. In PBMC-CRYO samples, the pDC/mDC ratio

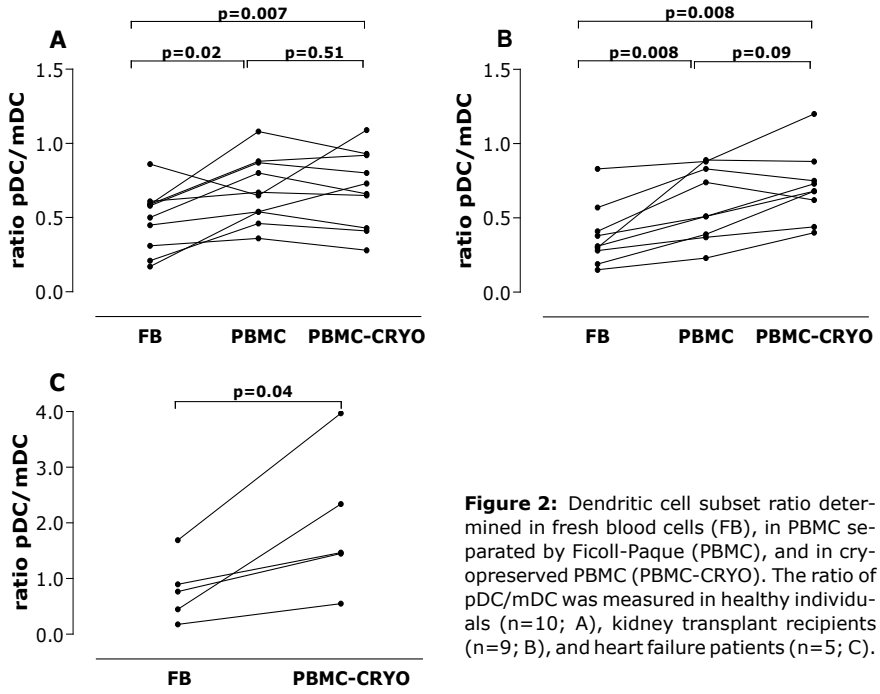

Figure 2: Dendritic cell subset ratio determined in fresh blood cells (FB), in PBMC separated by Ficoll-Paque (PBMC), and in cryopreserved PBMC (PBMC-CRYO). The ratio of pDC/mDC was measured in healthy individuals (n=10; A), kidney transplant recipients (n=9; B), and heart failure patients (n=5; C).

(median, 0.70; range, 0.28-1.1) was comparable to PBMC (p=0.51), but higher (p=0.007) than in corresponding unmanipulated fresh blood samples.

Also in KTx recipients, the pDC/mDC ratio was higher in PBMC (median, 0.51; range, 0.23-0.89) than in corresponding fresh blood samples (median, 0.31; range, 0.15-0.83; p=0.008) (Figure 2B). In PBMC-CRYO samples, the pDC/mDC ratio (median, 0.68; range, 0.40-1.2) increased further in comparison to Ficoll-isolated PBMC (p=0.09).

To investigate whether the increase in pDC/mDC ratio after cryopreservation in KTx recipients was due to immunosuppressive drugs, pDC/mDC ratio was also determined in HF patients, not treated with immunosuppression. The pDC/mDC ratio in PBMC-CRYO of HF patients was also higher (median, 1.5; range, 0.55-3.97) than the corresponding unmanipulated blood samples (median, 0.77; range, 0.18-1.69; p=0.04; Figure 2C).

Analysis of proportion mDC and pDC in unmanipulated cells, Ficoll-isolated and cryopreserved PBMC showed that manipulation of PBMC resulted in higher proportion of both mDC and pDC compared to unmanipulated cells in both groups (Table 4). After Ficoll-isolation of PBMC in healthy individuals, we found approximately 3-fold increase in proportion of both mDC and pDC compared to fresh blood cells. However, cryopreservation of PBMC resulted in a 5-fold increase in proportion of both mDC and pDC in comparison to unmanipulated cells. In KTx recipients, we found a 4-fold increase in proportion of both mDC and pDC after Ficoll-isolation of PBMC in comparison to fresh blood cells. Cryopreserved PBMC resulted in a 6-fold and 8-fold increase in proportion of mDC and pDC, respectively, compared to unmanipulated cells.

Table 4: Influence of Ficoll-Paque separation and PBMC cryopreservation on proportion pDC and mDC in comparison to fresh blood cells

	Proportion mDC (%)			**Proportion pDC (%)**			**n-fold increase in mDC compared to FB**		**n-fold increase in pDC compared to FB**	
	[3]FB	[4]PBMC	[5]CRYO	FB	PBMC	CRYO	PBMC:FB	CRYO:FB	PBMC:FB	CRYO:FB
HI1[1]	0.26	0.81	0.91	0.06	0.37	0.37	3.1	3.5	6.2	6.2
HI2	0.19	0.78	0.77	0.11	0.69	0.71	4.1	4.1	6.3	6.5
HI3	0.22	1.06	1.35	0.11	0.85	0.89	4.8	6.1	7.7	8.1
HI4	0.26	1.09	1.31	0.05	0.59	0.56	4.2	5.0	11.8	11.2
HI5	0.15	0.79	0.92	0.09	0.85	0.86	5.3	6.1	9.4	9.6
HI6	0.17	0.52	0.57	0.10	0.45	0.45	3.1	3.4	4.5	4.5
HI7	0.29	0.84	0.62	0.13	0.45	0.45	3.0	2.1	3.5	3.5
HI8	0.07	0.43	0.43	0.06	0.28	0.47	6.1	6.1	4.7	7.8
HI9	0.32	0.95	1.13	0.10	0.34	0.32	3.0	3.5	3.4	3.2
HI10	0.31	1.08	1.19	0.19	0.72	0.77	3.5	3.8	3.8	4.1
						median	**3.8**	**4.0**	**5.5**	**6.4**
KTx1[2]	0.12	0.75	0.61	0.10	0.66	0.73	6.3	5.1	6.6	7.3
KTx2	0.08	0.37	0.33	0.03	0.19	0.24	4.6	4.1	6.3	8.0
KTx3	0.20	0.93	0.72	0.03	0.21	0.29	4.7	3.6	7.0	9.7
KTx4	0.10	0.46	0.52	0.03	0.41	0.46	4.6	5.2	13.7	15.3
KTx5	0.16	0.70	0.63	0.05	0.36	0.43	4.4	3.9	7.2	8.6
KTx6	0.14	0.45	0.57	0.18	0.38	0.43	3.2	4.1	2.1	2.4
KTx7	0.24	0.85	0.88	0.07	0.32	0.39	3.5	3.7	4.6	5.6
KTx8	0.16	0.50	0.35	0.03	0.20	0.24	3.1	2.2	6.7	8.0
KTx9	0.31	0.97	1.40	0.13	0.72	0.87	3.1	4.5	5.5	6.7
						median	**4.4**	**4.1**	**6.6**	**8.0**

[1]HI, healthy individual; [2]KTx, kidney transplant recipient; [3]FB, fresh blood cells; [4]PBMC, Ficoll-isolated PBMC; [5]CRYO, cryopreserved PBMC.

Effect of Ficoll-Paque separation and cryopreservation of PBMC on percentage dendritic cell subsets positive maturation markers

From 6 out of 10 healthy individuals and 4 out of 9 KTx recipients, percentage (%) of mDC and pDC positive for CKR were determined (Figure 3). Because of the low numbers, the healthy individuals (n=6) and KTx recipients (n=4) were combined in one group.
In PBMC samples (median, 54.6%; range 15.2-99.87), %CCR7$^+$ mDC was higher than in both corresponding fresh blood samples (median, 15.0%; range, 8.3-46.0; p=0.03) and PBMC-CRYO samples (median, 11.6%; range, 1.3-94.2; p=0.005; Figure 3A). After PBMC-CRYO, %CCR5$^+$ mDC (median, 76.8%; range, 60.0-98.1) was lower compared to unmanipulated blood samples (median, 97.9; range, 82.4-99.4; p=0.03) and PBMC samples (median, 93.9%; range, 85.6-99.6; p=0.02; Figure 3B). For %CXCR4$^+$ mDC, comparable results were found as for CCR5$^+$ mDC (Figure 3C). Comparable results were found between %CCR5$^+$ mDC and CXCR4$^+$ mDC, because both CKR were analysed in the same dotplot (Figure 1C).

As for mDC, %CCR7$^+$ pDC was also higher in PBMC samples (median, 98.4%; range, 93.7-99.9) than in unmanipulated blood samples (median, 96.7%; range, 92.5-98.7; p=0.04) and in PBMC-CRYO (median, 96.7; range 93.5-98.3; p=0.03; Figure 3D). As shown

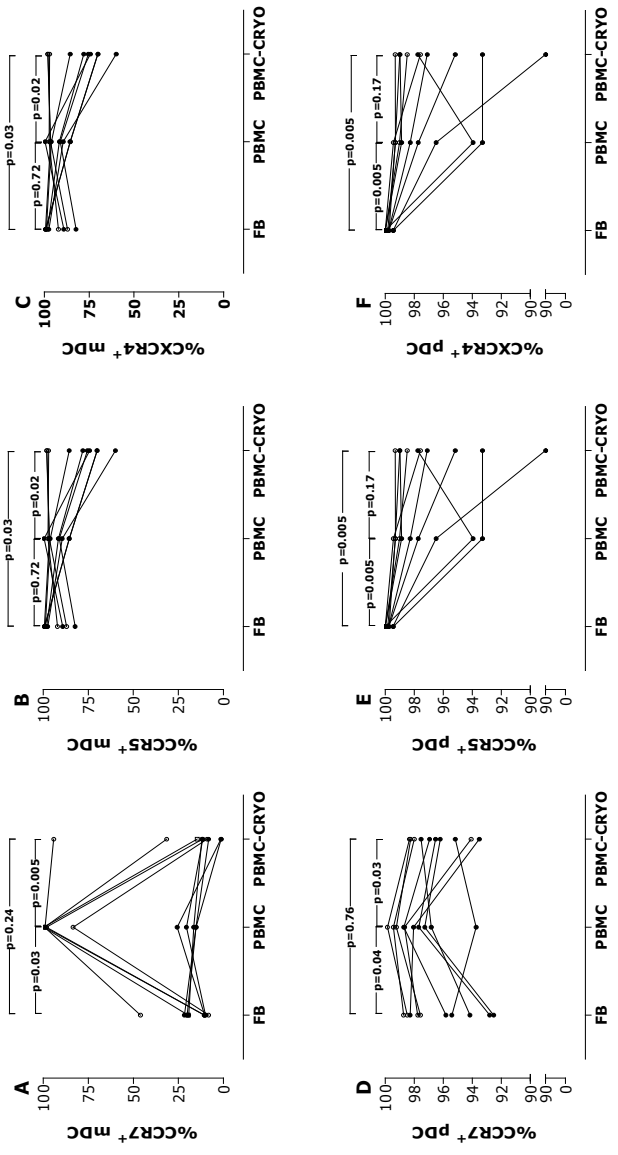

Figure 3: Percentage (%) of chemokine receptors CCR7 (A and D), CCR5 (B and E), and CXCR4 (C and F) on myeloid DC (mDC) and plasmacytoid DC (pDC), respectively, determined in fresh blood cells (FB), PBMC separated by Ficoll-Paque (PBMC), and cryopreserved PBMC (PBMC-CRYO) from 6 healthy individuals and 4 kidney transplant recipients. Black circles: healthy individuals; open circles: kidney transplant recipients.

in Figure 3E, %CCR5$^+$ pDC in fresh blood samples was higher (median, 99.8%, range, 99.4-100) than the corresponding PBMC (median, 98.6%; range, 93.3-99.4; p=0.005) and PBMC-CRYO (median, 97.7%; range, 89.6-99.3; p=0.005). Comparable results were observed for %CXCR4$^+$ pDC (Figure 3F).

Effect of Ficoll-Paque separation and cryopreservation of PBMC on chemokine receptor expression levels on dendritic cell subsets

The expression levels (gMFI) of CKR on DC subsets were also determined in 6 healthy individuals and 4 KTx recipients.

The expression of CCR7 on mDC was comparable between PBMC, PBMC-CRYO, and fresh blood samples (Figure 4A). In contrast, the expression of CCR5 on mDC was lower after cryopreservation (median, 56.6 gMFI; range, 8.6-95.4) compared to fresh blood samples (median, 69.9 gMFI; range, 36.5-114.9; p=0.01) and PBMC (median, 70.6 gMFI; range, 44.8-108.1; p=0.05; Figure 4B). In comparison to unmanipulated fresh blood samples, expression of CXCR4 on mDC showed no differences between PBMC samples and PBMC-CRYO (Figure 4C).

The expression of CCR7 on pDC was higher in PBMC samples (median, 45.4 gMFI; range, 18.6-76.5; p=0.005) and PBMC-CRYO (median, 43.0; range, 22.6-67.5; p=0.005) compared to unmanipulated blood samples (median, 26.7; range, 11.6-40.2; Figure 4D). A decrease in CCR5 expression on pDC was observed in PBMC samples (median, 53.8 gMFI; range, 28.2-85.1) compared to fresh blood samples (median 72.8 gMFI; range, 39.0-131.4; p=0.005; Figure 4E). CCR5 expression on pDC decreased even further after cryopreservation compared to PBMC (median, 27.8 gMFI; range, 16.8-61.7; p=0.005). Also, CXCR4 expression on pDC showed lower expression in PBMC samples (median, 46.9 gMFI; range, 12.3-62.5; p=0.005) and PBMC-CRYO (median, 39.1 gMFI; range, 6.0-236.1; p=0.007) compared to unmanipulated samples (median, 183.4 gMFI; range, 95.3-238.4; Figure 4F).

Some differences in the pDC population between the KTx recipients and healthy individuals were found. The percentage (%; Figure 3) pDC positive for CCR7 and expression (gMFI; Figure 4) of CCR7 on pDC were higher in fresh blood cells from KTx recipients than healthy individuals. The percentage pDC positive for CCR5 and CXCR4, and gMFI of CCR5 and CXCR4 on pDC were comparable between both groups. After Ficoll-isolation of PBMC, percentage pDC positive for CCR7, CCR5 and CXCR4, and expression of CCR7, CCR5 and CXCR4 on pDC were higher ($p<0.05$) in KTx recipients than in healthy individuals. After cryopreservation the percentage pDC positive for CCR7, CCR5 and CXCR4, and expression of CCR7, CCR5 and CXCR4 on pDC were comparable between the two groups.

DISCUSSION

In the present study, we demonstrate that Ficoll-Paque separation and subsequent cryopreservation of PBMC induces higher pDC/mDC ratios than the pDC/mDC ratio in fresh blood samples. Additionally, the biovariability within 3 weeks of the BDCA method shows that the CV of percentage pDC ranged between 3.0% and 17.9%, and percentage mDC demonstrates a CV always lower than 12%. The higher variability in pDC can be explained by the lower number of pDC present in fresh blood compared to the higher mDC numbers. These results are in line with a study of Hackstein *et al.*[23] who found an average CV of 3.8%

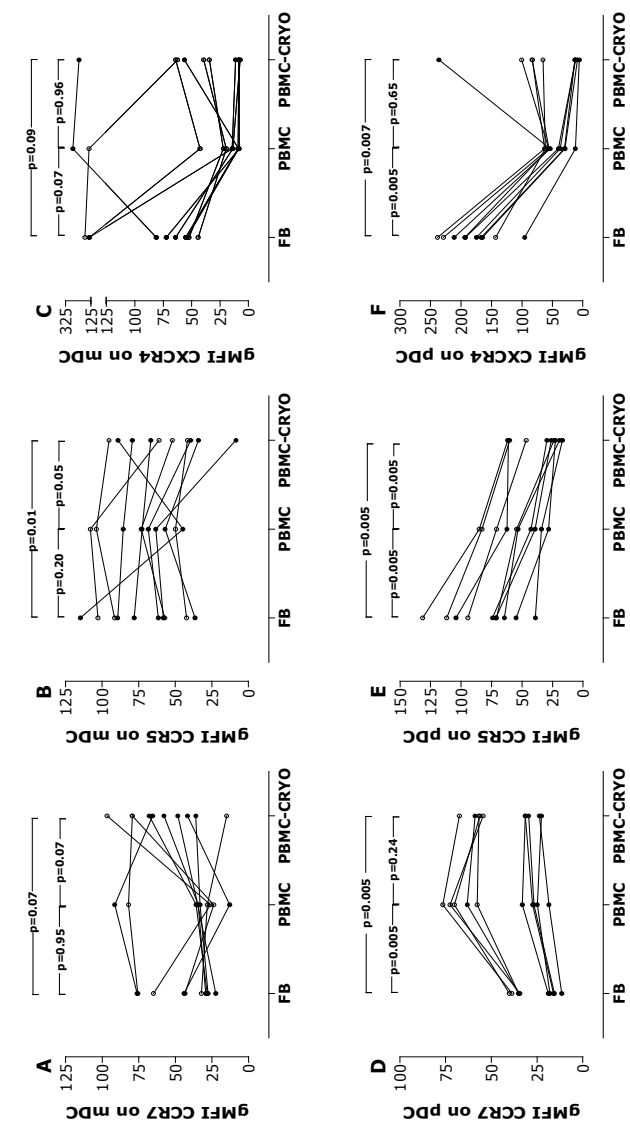

Figure 4: Expression of chemokine receptors CCR7 (A and D), CCR5 (B and E), and CXCR4 (C and F) on myeloid DC (mDC) and plasmacytoid DC (pDC), respectively, determined in fresh blood cells (FB), PBMC separated by Ficoll-Paque (PBMC), and cryopreserved PBMC (PBMC-CRYO) from 6 healthy individuals and 4 kidney transplant recipients. Black circles: healthy individuals; open circles: kidney transplant recipients. gMFI: geometric Mean Fluorescence Intensity.

for mDC and 2.9% for pDC, only for pDC we found higher CV.

The result of our study demonstrated that blood manipulations such as Ficoll-isolation and subsequent cryopreservation of PBMC influenced the proportion of both pDC and mDC. The relative large increase in pDC compared to mDC result in higher pDC/mDC ratio after blood manipulation compared to unmanipulated blood cells. KTx recipients received immunosuppression which is known to influence DC numbers, and DC development and differentiation.[20, 24, 25] To exclude that immunosuppression resulted in an additional effect on the pDC/mDC ratio, besides blood manipulations, the pDC/mDC ratio was measured in HF patients who did not receive immunosuppression. The results were comparable to those in KTx recipients, suggesting that higher pDC/mDC ratio in PBMC samples were caused by blood manipulations, and not by immunosuppressive drugs.

In peripheral blood, BDCA-1 (CD1c) antigen is expressed on human mDC, but also on B cells. In fresh blood, the identification of $CD14^{-}CD19^{-}BDCA\text{-}1^{+}$ mDC was performed as illustrated in Figure 1. However, Ficoll-isolation and cryopreservation of PBMC resulted in lower expression of CD19 on PBMC. As a consequence, B cells contaminated the $CD14^{-}$, $CD19^{-}$ fraction, resulting in a complex distinction in the identification of mDC compared to unmanipulated cells. In addition, lower CD14 expression on monocytes was observed after Ficoll-isolation and cryopreservation of PBMC in comparison to fresh blood cells, which made the analyses of DC subsets even more complicated. Therefore, in comparison to fresh blood cells, blood manipulation of PBMC resulted in an altered proportion of pDC and mDC not corresponding to the *in vivo* situation in healthy individuals and transplant recipients, thus causing incorrect interpretation of the results.

Recent studies showed a relation between the number of pDC and age in healthy individuals.[18, 23, 26] High numbers of pDC were found in individuals from 0-10 years of age, resulting in higher pDC/mDC ratio for young people.[18, 23, 26] Vuckovic *et al.*[18] showed that young individuals (<10 years) also have high number of mDC. In our study, all individuals tested were more than 24 years of age. Healthy individuals were younger than KTx recipients as well as HF patients, but all groups demonstrated comparable results with higher pDC/mDC ratio after blood manipulations. This suggests that age in this study probably did not affect the pDC/mDC ratio.

It has been suggested that the pDC/mDC ratio in peripheral blood might be useful for immunological monitoring in disease and organ transplantation.[17] For such monitoring purposes, we recommend that fresh blood cells should be used, because only this reflects the *in vivo* immune-status of transplant recipients. In multicenter trials, and trials with many patients, one has to keep in mind that only reliable results in pDC/mDC ratios will be obtained when using fresh blood cells. Similarly, other studies have also shown that lymphocyte subsets determination was more reliable in fresh blood than in Ficoll-isolated PBMC.[27, 28] The number of B cells and CD8+ cells were lower in Ficoll-isolated PBMC than in fresh blood cells, and the number of NK cells was higher after Ficoll-isolation of PBMC. Therefore, our results support the idea that both DC, as other lymphocyte subsets, should be measured in fresh blood for immunological monitoring purposes.

DC require signals to induce their differentiation and activation.[1] We examined the influence of Ficoll-isolation and cryopreservation of PBMC on maturation markers based on CKR. Those blood manipulations resulted in a shift towards mature DC by higher $\%CCR7^{+}$ mDC and $\%CCR7^{+}$ pDC. Additionally, lower $\%CCR5^{+}$, $CXCR4^{+}$ mDC and $\%CCR5^{+}$, $CXCR4^{+}$ pDC were found, suggesting that Ficoll-isolation and cryopreservation affects DC differentiation.

Costantini *et al.*[29] analysed the effect of cryopreservation on lymphocyte immunophenotype in healthy individuals and HIV-infected individuals. They found that

cryopreservation and Ficoll-isolation of PBMC decreased the %CCR5+ T cells compared to fresh whole blood. In contrast, cryopreservation and Ficoll-isolation did not affect the expression of CXCR4+ T cells. A study of Shalekoff and Tiemessen[30] reported higher %CXCR4+ cells and lower expression of CCR5 on leukocytes after overnight incubation with fluorescent-monoclonal antibody than after staining within 6 hours. This also suggests that most CKR are sensitive to small blood manipulations, and should therefore be measured in fresh blood samples within 4 h of blood sampling.

Recent studies analysed the DC maturation status after cryopreservation.[31, 32] John *et al.*[31] showed that CD83 expression was higher after cryopreserved cultured immature DC compared to fresh cultured immature DC, suggesting a phenotypically mature DC. On the other hand, Hori *et al.*[32] found no differences in the percentage CD83 either in cryopreserved cultured mature DC or in freshly cultured mature DC. We did not find differences between CD83 expression on DC after cryopreservation and CD83 expression after fresh blood samples (data not shown).

In conclusion, the current study shows that determination of pDC/mDC ratio and DC subset maturation status based on CKR expression is strongly influenced by Ficoll-Paque separation and subsequent cell cryopreservation. We recommend that only fresh blood samples should be used for monitoring purposes in both patient and control subjects.

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Part II

Tapering of Immunosuppression after HLA-Identical Living-Related Kidney Transplantation

Chapter 7

Granzyme B Elispot assay determines the cytotoxic T-lymphocyte precursor frequency after HLA-identical living-related kidney transplantation

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ABSTRACT

Background: A major goal in organ transplantation is to define the optimal immunosuppressive dose. Recently, we demonstrated that the frequency of cytotoxic T lymphocytes (CTLpf) identifies patients in whom the immunosuppressive load can be safely reduced. However, in peripheral blood mononuclear cells (PBMC) from HLA-identical living-related (LR) kidney transplant patients, no donor-specific CTLpf can be measured. The determination of the functional activity of cytotoxic T lymphocytes (CTL) could be an alternative method for the CTLpf. Granzyme B (GrB) is present in the granules of CTL and is involved in the direct lethal hit of donor target cells. Therefore, we wondered whether the GrB Elispot assay is an alternative method to determine the activity of CTL after HLA-identical LR kidney transplantation.

Methods: We measured the number of GrB producing cells (pc) against donor PBMC and 3rd-party PBMC in PBMC from HLA-identical patients who were reduced in their immunosuppression from 100% to 50% AZA with 5 to 10 mg/day prednisone.

Results: We found low numbers of GrB pc before reduction of immunosuppression, as only 20% of the patients' PBMC responded to donor cells, whereas 57% of the patients' PBMC responded to donor cells after reduction of immunosuppression. After 3rd-party stimulation, the number of GrB pc increased after tapering the immunosuppressive load ($p=0.03$).

Conclusion: Our results demonstrate that the GrB Elispot assay might be used as an alternative for the CTLpf after HLA-identical LR kidney transplantation.

INTRODUCTION

After organ transplantation most patients receive a standard dose of immunosuppression to prevent acute rejection. However, the chronic use of immunosuppression may result in several side-effects, such as infection, cancer, and diabetes mellitus.[1] Therefore, it is warranted to minimize the immunosuppressive load, provided this is not accompanied by acute rejection.

In the HLA-identical living-related (LR) kidney transplant setting, the HLA molecules are identical between patient and donor. However, differences may exist in the expression of minor histocompatibility antigens (mHAgs). mHAgs are peptides derived from polymorphic intracellular proteins presented in the context of HLA molecules that can be recognized by the immune system.[2] The clinical relevance of mHAgs after clinical transplantation is not known. After HLA-identical LR kidney transplantation, mHAgs might be responsible for alloreactivity as has been shown for graft-versus-host after identical bone marrow transplantation.[3]

Recently, we showed that kidney transplant patients with low donor-specific cytotoxic T lymphocyte precursor frequencies (CTLpf) could be safely reduced in their immunosuppression without the occurrence of rejection.[4] However, in HLA-identical LR kidney transplant patients, no donor-specific CTLpf can be measured. The functional activity of cytotoxic T lymphocytes (CTL) might be an alternative for the CTLpf that determines the cytolytic activity of CTL.[5] Granzyme B (GrB) is a relevant surrogate marker for functional activity of CTL. GrB is present in the granules of CTL and is involved, together with perforin, in inducing cell death in donor target cells through cleavage at specific aspartate residues resulting in mitochondrial damage.[6] We wondered whether the GrB Elispot assay is an alternative for determining the functional activity of CTL after HLA-identical LR kidney transplantation. In a pilot study, we determined the number of GrB pc in peripheral blood mononuclear cells (PBMC) from HLA-identical LR kidney transplant patients who were reduced from 100% azathioprine (AZA) in two steps to 50% AZA in combination with standard dose prednisone.

MATERIALS AND METHODS

Patients and PBMC sampling

GrB Elispot assays were performed in PBMC from seven patients who were more than 2 years post HLA-identical LR kidney transplantation (median, 4.6 years; range, 2.4-10.1). The patients were tapered from 100% AZA (median, 1.7 mg/kg; range, 1.1-1.8) in two steps to 50% AZA (median, 0.7 mg/kg; range, 0.5-1.1) in combination with 5 to 10 mg/day prednisone (Pred). Not enough PBMC were present in two patients to determine the number of GrB pc at 100% AZA. PBMC were isolated from heparinised blood by density gradient centrifugation using Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) as described before[4] and stored at −140°C until use.

Proliferation assay

The phytohemagglutinin (PHA) proliferation assay was performed to control viability of PBMC.[7] Only viable cells, according to this stimulation assay (stimulation index: ratio of the counts per minute obtained in the presence of PHA to the count per minute in the absence

of PHA ≥ 50), were analysed in the described results.

GrB Elispot assay

In a 96-well round bottom plate, 100 µl of 2x10^5 patients' PBMC in complete culture medium[7] was added to 100 µl of 2x10^5 irradiated (40 Gy) PBMC derived from the donor. In addition, 100 µl of 1x10^5 patients' PBMC was added to 100 µl of 1x10^5 irradiated (40 Gy) 3rd-party PBMC. To control the influence of irradiation on cytokine production, 1x10^5 patients' PBMC were incubated with 1x10^5 irradiated (40 Gy) patients' PBMC. If responses were found in this control, it was subtracted from the donor-reactive and 3rd-party response. The GrB Elispot assay was performed according to manual from the Elispot kit (Sanquin, Amsterdam, The Netherlands).

Statistical analysis

The Mann Whitney U-test was used to compare the patients who received 100% AZA with those who received 50% AZA. Two sided p-values ≤ 0.05 were considered significant.

RESULTS AND DISCUSSION

As shown in Figure 1A, before reduction of immunosuppression a median frequency of 0 GrB pc/10^6 PBMC (range: 0-80) was found after stimulation with donor PBMC. After tapering the immunosuppression to 50% AZA, a median frequency of 35 GrB pc/10^6 PBMC was found (range: 0-150; p=0.27). Interestingly, before the reduction of immunosuppression, only one of five (20%) of the HLA-identical patients responded against donor PBMC, whereas four of seven (57%) PBMC from those patients responded against donor PBMC after reduction of

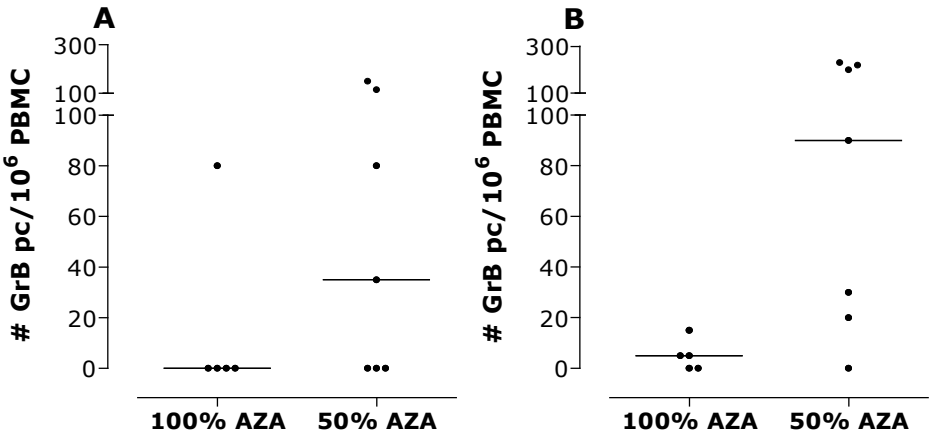

Figure 1: Number of donor-reactive (A) and 3rd-party (B) reactive granzyme B (GrB) producing cells (pc) in PBMC from HLA-identical living-related kidney transplant patients. Reactivity was measured before (100% AZA; n=5) and after (50% AZA; n=7) tapering of immunosuppression by using a GrB Elispot assay.

immunosuppression.

We also observed before reduction of immunosuppression, that three of five (60%) of the patients' PBMC responded to 3rd-party PBMC. The median frequency of the whole group before tapering of immunosuppression was 5 GrB pc/10^6 PBMC (range: 0-15) (Figure 1B). After tapering of immunosuppression, six of seven (86%) of the patients' PBMC responded to 3rd-party PBMC. The median of the whole group after tapering of immunosuppression was 90 GrB pc/10^6 PBMC (range: 0-230; p= 0.03).

Our results demonstrate that after HLA-identical LR kidney transplant patients, GrB pc can be determined mainly after reduction of immunosuppression. We assume that these responses are directed toward the mismatched donor mHAgs, and that the GrB Elispot assay can be used as an alternative for the CTLpf after HLA-identical LR kidney transplantation. Other groups used the interferon-γ (IFN-γ) Elispot assay as marker for the number of CTL.[8, 9] Recently, we demonstrated that IFN-γ pc specific for donor mHAgs can also be found after HLA-identical LR kidney transplantation.[7] In the future, we will determine the number of IFN-γ pc and GrB pc after tapering immunosuppression in HLA-identical LR kidney transplant patients to validate the clinical relevance of both Elispot assays.

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Chapter 8

Stable T-cell reactivity after successful tapering of azathioprine in HLA-identical living-related kidney transplant recipients despite minor histocompatibility antigens

Stable T-cell reactivity after successful tapering of azathioprine

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ABSTRACT

Background: Human leucocyte antigen (HLA)-identical living-related (LR) kidney transplant recipients often receive the standard regimen of immunosuppression. We wondered whether these patients should be exposed to the side effects of these drugs any longer. Safe tapering of immunosuppression should not result in rejection and high donor-directed T-cell responses. In the present study, we investigated the effect of tapering azathioprine (AZA) on T-cell reactivity.

Methods: Fifteen HLA-identical LR kidney transplant recipients receiving a median of 150 mg/day AZA and 5-10 mg/day prednisone were tapered to a median of 50 mg/day AZA. Donor, 3rd-party, and tetanus toxoid (TET)-reactivity were determined in interferon (IFN)-γ and interleukin (IL)-13 Elispot assays, which reflects the T-helper (Th)1 and T-helper (Th)2 response.

Results: After tapering of AZA, none of the patients developed acute rejection and the renal function remained stable, even at 1-year follow-up. The frequency of donor-reactive IFN-γ and IL-13 producing cells (pc) was low. Tapering of AZA did not influence the frequency of both IFN-γ and IL-13 pc. Also, the reactivity against 3rd-party cells and TET remained unchanged.

Conclusions: The AZA dose can be safely reduced in recipients of an HLA-identical LR kidney transplant without affecting kidney function and without increasing T-cell responses directed against donor or other antigens.

INTRODUCTION

Organ transplantation has become the preferred treatment for end-stage kidney function. To prevent graft rejection, transplant recipients receive immunosuppressive drugs to suppress immune responses directed to the foreign organ. Generally, lifelong administration of immunosuppressive drugs after transplantation is deemed necessary. However, chronic use of immunosuppression in transplant recipients is associated with several serious side effects, such as infections, osteoporosis, diabetes mellitus, and cancer.[1, 2] Consequently, discontinuation of immunosuppression is warranted, provided this is not accompanied by rejection.[3-5]

In the human leucocyte antigen (HLA)-identical living-related (LR) kidney transplant setting, the major HLA molecules A, B, C, DR, DQ, and HLA-DP are identical. However, mismatches may exist in minor histocompatibility antigens (mHAgs). The mHAgs are peptides derived from polymorphic intracellular proteins presented in the context of HLA molecules, which can be recognized by the immune system.[6-10] The expression of mHAgs is ubiquitous or specific on haematopoietic cells.[11]

In humans, mismatches in mHAgs have been shown to induce graft-*versus*-host disease (GVHD) after HLA-identical bone marrow transplantation (BMT). Goulmy *et al.* demonstrated that one or more disparities of HA-1, HA-2, HA-4, and HA-5 were associated with the development of GVHD.[12] After BMT, male recipients who received an HLA-identical graft from a female donor are more at risk in developing GVHD than those who received a graft matched for gender.[13] This risk is due to an enhanced activity against mHAgs HY encoded on the Y chromosome. Studies have shown the existence of HY specific cytotoxic T lymphocyte (CTL) after gender-mismatched BMT.[14]

The role of mHAgs in clinical solid organ transplantation is yet not known. Recently, Opelz described an association between the presence of panel reactive antibodies (PRA) in kidney transplants from HLA-identical sibling donors and long-term graft loss.[15] Theoretically, after HLA-identical LR kidney transplantation, mHAgs, but also other non-HLA antigens[15, 16], might be responsible for the induction of acute or chronic rejection of the transplant. Therefore, HLA-identical LR kidney transplant recipients still receive immunosuppression. Considering the adverse effects of immunosuppression, we questioned whether these patients should be exposed to the severe side effects of immunosuppression.

After HLA-mismatched kidney transplantation, some studies reported a relation with increased T-cell reactivity in mixed lymphocyte culture (MLC), CTL precursor frequency (CTLpf), helper T lymphocyte precursor frequency (HTLpf), and acute rejection after tapering of immunosuppression.[17-20] However, in the HLA-identical LR kidney transplant setting, no donor-reactive responses can be measured in CTLpf, HTLpf and MLC. Recently, we have shown that a sensitive method as the Elispot assay can be used to determine the reactivity against non-HLA antigens in the first period after HLA-identical LR kidney transplantation.[21] The Elispot assay can be used to determine the frequency of pro-inflammatory cytokines (e.g. IFN-γ) and anti-flammatory cytokines (e.g. IL-13) directed to donor antigens. Both types of cytokines have been associated with acute rejection[22, 23], while Th2-cytokines also have been suggested with graft acceptance.[24, 25]

In the current study, HLA-identical LR kidney transplant recipients, more than 2 years after transplantation, were safely tapered to at least 50% of their azathioprine (AZA) dose. We questioned whether tapering the immunosuppressive load would result in increased T-cell reactivity in peripheral blood mononuclear cells (PBMC) from HLA-identical LR kidney transplant recipients. T-cell reactivity against donor, 3rd-party, and tetanus toxoid

(TET) antigens was retrospectively measured in IFN-γ and IL-13 Elispot assays.

SUBJECTS AND METHODS

Patients

HLA-identical LR kidney transplant recipients, treated with AZA and prednisone, were asked to participate in this study. They were at least 2 years after transplantation, had no acute rejection in the last 6 months, had stable renal function, and no clinical relevant proteinuria (≥ 0.5 g/l). Fifteen patients agreed to participate. Characteristics of the patients are described in Table 1. At inclusion of the study, the patients received a median dose of 150 mg/day AZA (range, 100-175 mg/day; 'high AZA') in combination with a median dose of 10 mg/day prednisone (range, 5-10 mg/day; Pred). The AZA dose was tapered twice with a four months interval to a median dose of 50 mg/day AZA (range, 50-100 mg/day; 'low AZA'). The prednisone dose remained unchanged. Peripheral blood samples were analyzed at inclusion and 4 months after the last mentioned AZA dose reduction. Renal function was closely monitored.

PBMC Sampling

PBMC were isolated from heparinized blood by density gradient centrifugation using Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) as described before.[21] Thereafter, PBMC were stored in RPMI-1640-DM (BioWhittaker, Verviers, Belgium) containing 15% fetal calf serum (FCS) and 10% dimethyl sulfoxide (MERCK, Germany) at –140°C until use.

IFN-γ and IL-13 Elispot assay

The phytohemagglutinin (PHA) proliferation assay was performed to control the viability of the PBMC as described before.[21] The mean counts per minute (cpm) were determined, and the stimulation index (SI) was calculated by the ratio of the cpm obtained in the presence of PHA to the cpm in the absence of PHA. Only results of viable cells (SI ≥ 50) were analyzed in the described results.

Before the first tapering of the immunosuppressive medication, we received 35 ml heparinized peripheral blood from 15 patients. Four months after the last AZA dose reduction, we received again 35 ml blood from those patients to perform Elispot assays. The IFN-γ and IL-13 Elispot assays (U-CyTech Biosciences, Utrecht, The Netherlands) were performed as described before.[21] Briefly, in a 96-well round bottom plate (Nunc, Roskilde, Denmark), 100 µl of $2x10^5$ patients' PBMC in culture medium was added to 100 µl of $2x10^5$ irradiated (40 Gy) PBMC derived from the donor. As control, unstimulated responder cells were used and subtracted from donor-reactive responses. PBMC from the patient were also stimulated with 1 µg/ml PHA (Murex Biotech), 3rd-party cells and 30 lf/ml tetanus toxiod (TET, RIVM, Bilthoven, The Netherlands). PHA was used as a positive control and showed in all experiments more than 100 spots per well. The 3rd-party PBMC used did not share HLA antigens with donor and patient. Both before and after tapering of immunosuppression, the same 3rd-party PBMC were used. TET stimulation was used to test the immune response to nominal antigens. After 40 h of incubation, the non-adherent cells were transferred to a 96-well plate pre-coated with anti-human IFN-γ or IL-13 monoclonal antibodies. The cells

Table 1: Characteristics of HLA-identical living-related kidney transplant recipients more than two years after transplantation

Pat[1]	Gender		Relation	Primary disease	Time after Tx[5] (years)	CMV[3]		EBV[4]		Before tapering ('high AZA')		After tapering ('low AZA')	
	Pat	Don[2]				Pat	Don	Pat	Don	AZA[7] (mg/d[9]) / (mg/kg[10])	Pred[8] (mg/d)	AZA[7] (mg/d) / (mg/kg)	Pred[8] (mg/d)
1	female	female	mother-daughter	Rapidly progressive glomerulonephritis	2.4	+	-	-	+	150 / 1.9	10	50 / 0.65	10
2	male	female	brother-sister	IgA nephropathy	5.0	+	+	+	+	150 / 1.6	10	100 / 1.09	10
3	female	female	daughter-mother	Focal segmental glomerulosclerosis	10.1	-	-	-	-	125 / 1.71	5	50 / 0.67	5
4	male	male	brother-brother	Membranous glomerulonephritis	4.5	-	+	-	+	150 / 1.88	7.5	50 / 0.67	7.5
5	female	female	sister-sister	Medullary cystic kidney disease	4.8	-	+	+	+	100 / 1.30	10	50 / 0.66	10
6	male	female	brother-sister	Autosomal dominant polycystic kidney disease	4.3	+	-	+	+	100 / 1.10	7.5	50 / 0.57	7.5
7	male	female	brother-sister	Acute tubular hypertension	5.2	-	+	+	+	125 / 1.84	10	50 / 0.75	10
8	male	female	brother-sister	Membranous glomerulonephritis	6.7	+	+	+	+	150 / 1.74	5	50 / 0.56	5
9	female	female	sister-sister	Hypertension	15.2	-	+	-	ND[6]	100 / 0.95	5	50 / 0.49	5
10	male	male	brother-brother	IgA nephropathy	4.9	+	-	+	+	175 / 1.58	7.5	75 / 0.65	7.5
11	male	female	brother-sister	Von Hippel Lindau	3.4	+	+	+	+	150 / 1.74	10	50 / 0.61	10
12	female	male	sister-brother	Extracapillary glomerulonephritis	3.0	-	-	+	ND	150 / 2.17	5	75 / 1.04	5
13	female	female	sister-sister	Chronic pyelonephritis	10.4	-	+	+	ND	100 / 1.41	5	50 / 0.68	5
14	male	female	brother-sister	Reflux nephropathy	3.7	+	+	+	ND	150 / 1.58	10	50 / 0.51	10
15	male	female	brother-sister	Focal segmental glomerulosclerosis	4.7	+	+	+	+	175 / 1.70	10	100 / 0.93	10
				Median	4.9					150 / 1.70	10	50 / 0.66	10

[1]Pat, patient; [2]Don, donor; [3]CMV, cytomegalovirus; [4]EBV, Epstein-Barr virus; [5]Tx, transplantation; [6]ND, not documented; [7]AZA, azathioprine; [8]Pred, prednisone; [9]mg/d, mg/day; [10]mg/kg, mg/kg body weight.

were incubated for 5 h at 37°C to allow the formation of spots. The spots were counted automatically by using a Bioreader 3000 Elispot reader (BioSys, GmbH, Karben, Germany).

Minor histocompatibility antigens typing after HLA-identical living-related kidney transplantation

Patient PBMC and donor PBMC were typed for 10 known mHAgs: H-Y, HA-1, HA-2, HA-3, HA-8, HB-1, BCL2A1 [encoded by two separate single nucleotide polymorphisms (ACC-1 and ACC-2) on a single gene], HwA-9, and UGT2B17 (Table 2). DNA from the selected couples (Table 1) was isolated using the QIAamp® DNA Mini Kit. The polymerase chain reaction (PCR-SSP) technique was used as typing method.[26] This is a PCR-based technique with the use of Sequence Specific Primers, which shortens the post-amplification processing time to a simple gel electrophoresis detection step. Each tested mHAgs is a part of a di-allelic system. This means that each locus contains two alleles. We used a specific primer combination for each allele, to test the presence of this allele (Table 2).

Statistical analysis

Numerical data were compared using the Wilcoxon signed rank test. Two sided p-values ≤ 0.05 were considered significant. For statistical analysis GraphPad statistical program was used (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Clinical results

After tapering the AZA dose to at least 50% of the original dose, none of the HLA-identical LR renal transplant recipients developed acute rejection. Their serum creatinine levels remained unchanged before and 4 months after halving the AZA dose (before tapering: median, 103 μmol/l; range, 63-128; after tapering: median, 102 μmol/l; range, 63-136). At 1-year follow-up, no clinical relevant increase in serum creatinine (median, 111 μmol/l; range, 67-138) and no proteinuria (≥0.5 g/l) had occurred. Additionally, haematological parameters [haemoglobline, haematocrite, mean cell volume (MCV), thrombocytes and leucocytes] remained normal during tapering of AZA (data not shown).

IFN-γ Elispot

Before tapering of AZA ('high AZA'), and 4 months after AZA-reduction ('low AZA'), 11 out of 15 (73%) HLA-identical patients responded to donor antigens (Figure 1A). The median frequency of IFN-γ producing cells (pc) directed to donor antigens after reduction of AZA (median, 1 IFN-γ pc/2x10^5 PBMC; range, 0-110; p=0.99) was comparable with before dose reduction (median, 2 IFN-γ pc/2x10^5 PBMC (range, 0-88).

Before reduction of AZA, 13 out of 15 (87%) patients responded to HLA-mismatched 3rd-party PBMC. After tapering to low AZA, 15 out of 15 (100%) patients responded to 3rd-party antigens (Figure 1B). The median frequency of IFN-γ pc directed to 3rd-party antigens after tapering of AZA (median, 12 IFN-γ pc/2x10^5 PBMC; range, 2-324; p=0.95) was comparable with before tapering (median, 14 IFN-γ pc/2x10^5 PBMC; range, 0-305). The

Table 2: Characteristics of minor histocompatibility antigens (mHAgs) and primer sequences

mHAgs[1]	Position chromosome	Pos[2] allele	Peptide	Neg[3] allele	Polymorphism	5′ primer sequences[4]	3′ primer sequences
HA-1	19	H	VLHDDLLEA	R	H	CTT AAG GAG TGT GTG CTG CA	ACT CCT ACA CAT CCC TCA GA
					R	CTT AAG GAG TGT GTG TTG CG	
HA-2	7	V	YIGEVLVSV	M	V	ACA GTC TCT GAG TGG CTC AG	GCT CCT GGT AGG GGT TCA C
					M		GCT CCT GGT AGG GGT TCA T
HA-3	15	T	VTEPGTAQY	M	T	CTT CAG AGA GAC TTG GTC AC	GTT CAT GAG CCC ATG TTC CAT
					M	CTT CAG AGA GAC TTG GTC AT	AGA CTC AGC AGG TTT GTT AC
HA-8	9	R	RTLDKVLEV	P	R	TGC AGT CAG CAG ATC ACC G	CTT CTG GGC AAC AGT TAT GGA
					P	TGC AGT CAG CAG ATC ACC C	
HB-1	5	H	EEKRGSLHV W	Y	H	GCC ATT CTT TTC TAT AGG TTC TTT GT	AGG GCA TAT GTT CCA CTT GCT T
					Y	ATT CTT TTC TAT AGG TTC TCT GC	
BCL2A1 (ACC-1)	15 AZ19	Y	DYLQYVLQI	C	Y	CAT TGC CTC AAC AGC TTC AAG	GGT TGT GGT ATC TGT AGG GCG T
					C		GGT TGT GGT ATC TGT AGG GCG C
BCL2A1 (ACC-2)	15 AZ82	D	KEFEDDIINW	G	D	GAT GGA AAA GGA GTT TGA AGG CGA	CAG CCT CCG TTT TGC CTT ATC
					G	GAT GGA AAA GGA GTT TGA AGG CGG	
HwA-9	2	R	RVWDLPGVL K	G	R	AAT GTG GTT TGA AGA CCA AAA GT	C TTG TAC TCT CAT CTT ACC TCT
					G		C TTG TAC TCT CAT CTT ACC TCC
UGT2B17	4	+	AELLNIPFLY	-	Ex1a	TGT GTT GGG AAT ATT CTG ACT ATA A	CCC ACT TCT TCA GAT CAT ATG CTT
HY	Y	+		-		TGG CGA TTA AGT CAA ATT CGC	CCC CCT AGT ACC CTG ACA ATG TAT T

[1]mHAgs, minor histocompatibility antigens; [2]Pos, positive; [3]Neg, negative; [4]Primer sequences were kindly provided by E. Spierings from Leiden, The Netherlands.

response to HLA-mismatched 3rd-party antigens was significantly higher than the response to donor antigens ($p<0.01$).

Before tapering AZA dose, we found that 13 out of 15 (87%) patients responded to TET. The median frequency of IFN-γ pc directed to TET antigens before tapering AZA was 7 IFN-γ pc/2x10^5 PBMC (range, 0-1200). After tapering to low AZA, 11 of the 15 patients (73%) responded to TET, and tapering of AZA did not influence the number of IFN-γ pc (median, 3 IFN-γ pc/2x10^5 PBMC; range, 0-1200; $p=0.38$).

Some of the HLA-identical patients had high responses against donor-PBMC in the IFN-γ Elispot assay (≥ 10 IFN-γ pc/2x10^5 PBMC; high responders). We analyzed whether other parameters could clarify the differences between high responders and nonresponders (0 IFN-γ pc/2x10^5 PBMC). Haematological parameters, transaminases, and renal function (proteinuria and creatinine clearance) were comparable between high responders and nonresponders.

After 3rd-party stimulation, we observed in four patients (patient 3, 4, 7 and 15) high frequencies of IFN-γ pc before and/or after tapering of AZA (> 45 IFN-γ pc/2x10^5 PBMC; Figure 1B). The response against donor antigens in two of the four patients (patient 4 and 7) remained low and stable after tapering of AZA (Table 3). This could indicate that before and after tapering the AZA dose, the Th1 cells in those patients are still able to recognize foreign antigens other than those of the donor antigens of the kidney. It was remarkable that one of those four patients (patient 3) showed high 3rd-party responses and donor-reactive responses after tapering of AZA (Table 3). In this patient, renal function was stable during tapering of immunosuppression. Two patients (patient 6 and 15) demonstrated a decrease in 3rd-party antigens. The donor-reactive response also decreased significantly in one patient (patient 6), and slightly in the other patient (patient 15). Both patients showed

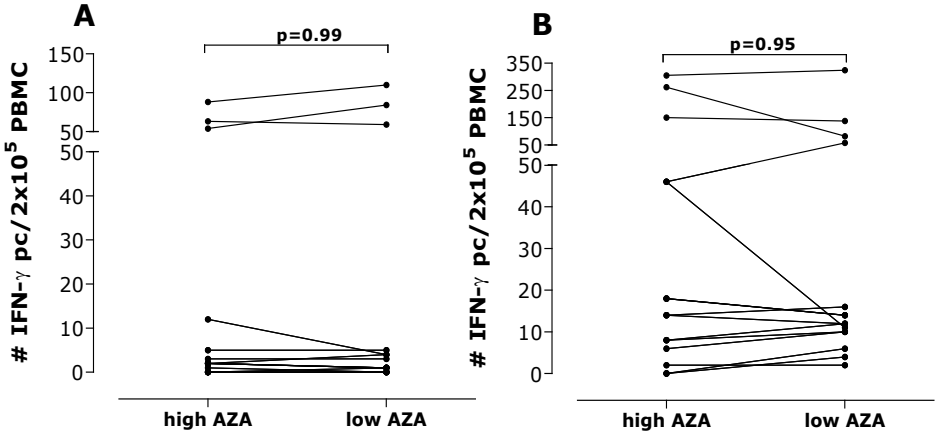

Figure 1: Number of IFN-γ producing cells (pc) reactive to donor cells (A) and 3rd-party cells (B) determined in PBMC from HLA-identical living-related kidney transplant recipients before and after tapering of AZA dose (n=15). (A) Donor-reactive response: during high AZA, median, 2 IFN-γ pc/2x105 PBMC (range: 0-88); during low AZA, median, 1 IFN-γ pc/2x105 PBMC (range: 0-110). (B) Third-party response: during high AZA, median, 14 IFN-γ pc/2x105 PBMC (range: 0-305); during low AZA, median, 12 IFN-γ pc/2x105 PBMC (range: 2-324). The Wilcoxon signed rank test was used to compare HLA-identical recipients before and after reduction of immunosuppression (donor, p=0.99; 3rd-party, p=0.95).

no abnormalities in renal function, liver function or haematological parameters.

IL-13 Elispot

As shown in Figure 2A, before reduction of AZA a detectable frequency of IL-13 pc against donor cells was observed in 8 out of 15 (53%) patients. After reduction to low AZA, only 4 out of 15 (27%) patients responded to donor antigens (p=0.26). The responses to donor antigens after tapering of AZA (median, 0 IL-13 pc/2x10^5 PBMC; range, 0-53; p=0.55) were comparable to before reduction (median, 1 IL-13 pc/2x10^5 PBMC; range, 0-30).

Before reduction of AZA, 12 out of 14 (86%) patients responded to 3rd-party antigens (Figure 2B). The median frequency of the whole group before tapering was 6 IL-13 pc/2x10^5 PBMC (range, 0-32). After tapering to low AZA, 10 out of 14 (71%) patients responded to 3rd-party antigens. Reduction of AZA also did not increase the number of IL-13 pc (median, 4 IL-13 pc/2x10^5 PBMC; range, 0-20; p=0.18). The response to 3rd-party antigens was significantly higher than the donor-reactive response (p=0.01).

Before and after reduction of AZA, 10 out of 14 (71%) patients responded to TET. No difference in the frequency of IL-13 pc to TET was found before (median, 2 IL-13 pc/2x10^5 PBMC; range, 0-187) and after (median, 2 IL-13 pc/2x10^5 PBMC; range, 0-127; p=0.46) tapering of AZA.

Similar to the IFN-γ Elispot result, patient 3 demonstrated high frequencies of IL-13 pc against donor antigens before (30 IL-13 pc/2x10^5 PBMC) and after (53 IL-13 pc/2x10^5 PBMC) tapering of AZA. Whether these high frequencies of IL-13 pc were caused by mismatches in mHAgs remains unknown, because no mismatches for known mHAgs were

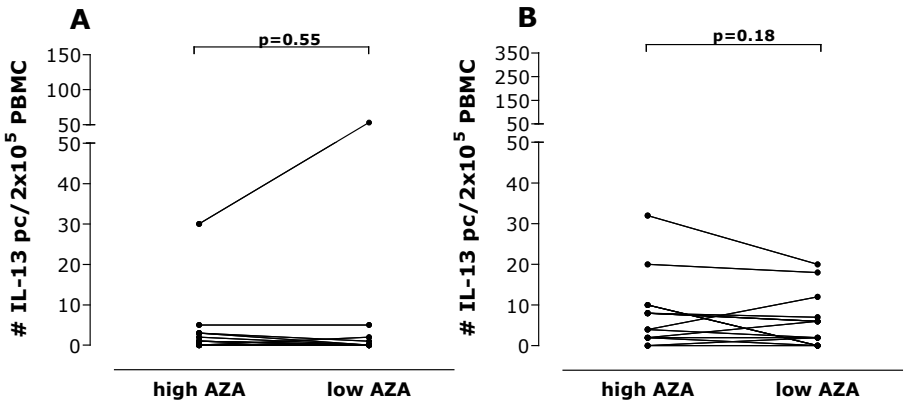

Figure 2: Number of IL-13 producing cells (pc) reactive to donor cells (A) and 3rd-party cells (B) determined in PBMC from HLA-identical living-related kidney transplant recipients before and after tapering of AZA dose (n=15). (A) Donor-reactive response: during high AZA, median, 1 IL-13 pc/2x105 PBMC (range: 0-30); during low AZA, median, 0 IL-13 pc/2x105 PBMC (range: 0-53). (B) Third-party response: during high AZA, median, 6 IL-13 pc/2x105 PBMC (range: 0-32); during low AZA, median, 4 IL-13 pc/2x105 PBMC; range: 0-20). The Wilcoxon signed rank test was used to compare HLA-identical recipients before and after reduction of immunosuppression (donor, p=0.55; 3rd-party, p=0.18).

found with the correct HLA-restriction element between donor and recipient (Table 3).

Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) after HLA-identical LR kidney transplantation

Table 1 shows CMV and EBV serology of donor and recipient. Before transplantation, eight patients were CMV-positive and seven patients were CMV-negative. From the CMV-negative patients, 5 out of 7 received a CMV-positive graft. Those 5 patients (CMV-/+) were not different in their cytokine-profile of IFN-γ and IL-13 from the other 10 patients (CMV +/+, CMV +/-, CMV -/-).

Eleven patients were EBV-positive and four patients were EBV-negative before transplantation. From the EBV-negative patients, 2 out of 4 received an EBV-positive graft, one patient received an EBV-negative graft, and from one patient the EBV serology was not documented. The donor-reactive cytokine profiles were comparable between those two 'EBV-negative patient EBV-positive donor' (EBV-/+) combinations and 11 EBV-positive patients.

Minor histocompatibility antigens typing after HLA-identical LR kidney transplantation

Patient PBMC and donor PBMC were typed for 10 known mHAgs that could cause a T-cell response (Table 2). We analyzed whether the IFN-γ and IL-13 Elispot results could be explained by mismatches in known mHAgs expressed with the correct restriction element.

In 11 out of 14 patient-donor combinations, we found mismatches for mHAgs (Table 3). The correct HLA-restriction element was present in only four patients. In 2 of these 4 patients, positive Elispot responses were found. In 6 out of the 7 patients without the correct HLA-restriction element, also positive Elispot results were found. Three patient-donor combinations were identical for the 10 determined mHAgs. Those patients had no significant response in the IFN-γ and IL-13 Elispots.

DISCUSSION

In the present study, we show that the immunosuppressive medication of HLA-identical LR kidney transplant recipients, who were more than 2 years after transplantation, can be safely reduced. Four months after the last AZA reduction to at least 50% of the original dose, no acute rejection or decrease in renal function had occurred. Also, at 1-year follow-up, the kidney function remained stable. Tapering of AZA did not increase the frequency of Th1 cells (IFN-γ) as well as the frequency of Th2 cells (IL-13).

The Elispot assay becomes an attractive method to determine donor-reactive responses in kidney transplant recipients and may be used to monitor patients. A study of Gebauer *et al.*[27] determined the frequency of IFN-γ pc in 11 stable HLA-mismatched renal allograft recipients, who were more than 18 months after transplantation, and found low frequencies of donor-reactive IFN-γ pc (1-45 IFN-γ pc/3x10^5 PBMC). Hricik *et al.*[28] found also low frequencies of alloreactive IFN-γ pc (<10 IFN-γ pc/3x10^5 PBMC) in renal allograft recipients with low serum creatinine levels within 6 months after kidney transplantation. Also after BMT with HLA-identical sibling donors, detectable IFN-γ pc were found in 20% of the donor cells stimulated with irradiated recipients cells.[29] During the current study, comparable frequencies of IFN-γ pc were found in the HLA-identical setting (high AZA: median, 2 IFN-γ pc/2x10^5 PBMC; range, 0-88; low AZA: median, 1 IFN-γ pc/2x10^5 PBMC; range, 0-110).

Table 3: Known mismatched minor histocompatibility antigens (mHAgs) and donor-reactive IFN-γ and IL-13 Elispot results

Patnr	Gender		Mismatched mHAgs in the HLA-identical donor	Restriction element present	IFN-γ ELISPOT[2]		IL-13 ELISPOT[2]	
	Patient	Donor			high AZA	low AZA	high AZA	low AZA
1	female	female	**HA-1R, HA-2V, HA-8R**[3]	A2	0	1	0	0
2	male	female	No known differences		0	0	3	0
3	female	female	HA-2M, BLC2A1Y		88	110	30	53
4	male	male	No known differences		2	1	0	0
5	female	female	HA-1H, HA-3M, HB-1Y		0	0	1	0
6	male	female	HA-3T, HwA-9G		12	4	1	0
7	male	female	HB-1Y		3	3	0	0
8	male	female	ND[1]		2	1	3	1
9	female	female	HB-1Y		5	5	2	0
10	male	male	No known differences		0	0	0	0
11	male	female	**HA-3M**, HA-8R, **HwA-9R**	A1, A3	1	0	0	0
12	female	male	**H-Y**, HA-3T	A2	2	1	5	5
13	female	female	BCL2A1Y, BCL2A1D		2	4	1	0
14	male	female	**HB-1H**	B44	54	84	0	0
15	male	female	HwA-9G		63	59	0	2

[1]Not determined; [2]Results are presented as the number of donor-reactive IFN-γ and IL-13 producing cells/2x105 PBMC; 3Mismatched mHAgs with the correct HLA-restriction element are presented as bold. Elispot results: negative = 0 IFN-γ or IL-13 pc; positive ≥ 1 IFN-γ or IL-13 pc.

It seems that those frequencies of IFN-γ pc are common in an immunological quiescence period after transplantation and is independent of HLA-matching.

The number of IFN-γ pc directed to HLA-mismatched 3rd-party cells and TET did not change after tapering of AZA, while those reactivities were significantly higher than the reactivity to donor antigens. This suggests that the immune response to nominal antigens, alloantigens as well as to donor antigens is not affected by the reduction of AZA. Therefore, our HLA-identical LR kidney transplant recipients are both clinically (stable kidney function, haematological parameters, and transaminases) and immunologically stable. These results are in line with our experience in patients who received a kidney transplant with one or more HLA-mismatches.[30] In this study also no increase in T-cell reactivity was found.

We also determined the Th2 response by using an IL-13 Elispot assay. IL-13 is an immunoregulatory cytokine secreted by activated Th2 cells.[24, 31] The importance of Th2 responses has been shown in several studies in acute as well as in chronic rejection.[23, 32, 33] In our study, we found that before and after tapering of AZA, 53% and 27%, respectively, of the patients' PBMC made IL-13 after stimulation with donor cells. Those frequencies of IL-13 pc were comparable before and after reduction of AZA. This suggests that the donor-reactive Th2 response, as well as the Th1 response, was not affected by reduction of AZA. The number of IL-13 pc after stimulation with 3rd-party PBMC and TET remained also in the same range before and after reduction of AZA, indicating that tapering does not affect both Th1 and Th2 reactivity towards foreign HLA and nominal antigens. Alternatively, the AZA in the given dose has no immunosuppressive effect on Th1 and Th2 response. In a pilot study, we found comparable frequencies of granzyme B (GrB) pc before and after tapering of AZA in PBMC from HLA-identical LR kidney transplant recipients, suggesting that the functional activity of CTL were also unaffected.[34] Unfortunately, not enough cells were available to test the frequency of GrB pc in this patient cohort.

In the present study, we observed that some of the HLA-identical patients demonstrated detectable (>10 IFN-γ pc/2x10^5 PBMC) frequencies of IFN-γ pc against donor antigens, whereas other patients had undetectable frequencies (0 IFN-γ pc/2x10^5 PBMC). No relation was found between IFN-γ frequencies and proteinuria, serum creatinine, CMV or EBV serology. Recently, Opelz *et al.*[15] showed an association of PRA-reactivity with long-term graft loss in kidney transplants from HLA-identical sibling donors. Those lymphocytotoxic antibodies could reflect high B-cell responsiveness and alternatively could be directed to mHAgs expressed on donor cells, but also to other non-HLA antigens.[16] Autologous responses are unlikely, because no difference was found between donor responses corrected for unstimulated patients' PBMC and donor responses corrected for the autologous control. Accordingly, in our study the donor-reactive Elispot responses could be directed to mismatches in mHAgs, but also to other non-HLA antigens.

Animal studies have shown that mHAgs are involved in the development of allograft arteriosclerosis, GVHD, and skin graft rejection.[35-38] In humans, the influence of mHAgs on transplant outcome have mostly been seen after BMT.[12, 39] Therefore, in our HLA-identical LR kidney transplant setting, mHAg disparities between patient and donor may theoretically trigger T-cell mediated rejection after tapering of immunosuppression. In our study, 14 patients were typed for 10 known mHAgs, which could give a T-cell response. Four of those patients had mismatches for mHAgs and the correct HLA restriction molecule. Only PBMC from 2 of the 4 patients had a T-cell response after donor stimulation. We also found that PBMC from 6 of the 7 patients with mismatches for known mHAgs, but not with the correct restriction element, had a donor-reactive T-cell response in the IFN-γ and/or IL-13 Elispot. A possibility for the response in those patient-donor combinations with mHAgs mismatches,

but not the correct HLA restriction, is that other HLA molecules can present the specific mismatched mHAgs. Therefore, not only mHAgs could be unknown, but also their HLA-restriction molecule. In theory, all genetic polymorphic proteins presented in MHC could give peptides recognized as mHAgs, but not all mHAgs are potential for inducing strong T-cell responses. The hierarchy of mHAgs could clarify the variety of the donor response. When a high donor response was found, immunodominant mHAgs could be present.[39, 40] Recently, Mori *et al.*[41] found that there is a hierarchy of immunodominance among mHAgs in mice, which is dependent on mHAgs density on host cells and the repertoire of donor T cells capable of responding to mHAgs. However, this phenomenon is unknown in humans. Mori *et al.*[41] suggested that GVHD is a consequence of activation of multiple T-cell populations against multiple mHAgs. In contrast, we found low numbers of IFN-γ and IL-13 pc towards multiple donor-mHAgs, and those were insufficient to induce rejection of the graft. This could indicate the presence of low affinity T cells recognizing donor mHAgs or that these mHAgs were not immunodominant. Therefore, these patients could be tolerant for the donor mHAgs. The last possibility could be that these patients are still over-immunosuppressed. After tapering of AZA, the patients received a median of 1.1 mg/kg AZA and 5-10 mg prednisone, and this may still be sufficient to prevent cytokine production of lymphocytes after donor-stimulation, and therefore rejection.

Some mHAgs as HA-1, HA-2 and BCL2A1 are only expressed on haematopoietic cells, while HA-3, HA-8, and H-Y are ubiquitously expressed.[14] Therefore, theoretically, T-cell responses could be found directed to those mHAgs only expressed on haematopoietic cells, while epithelial cells were not affected. Unfortunately, epithelial cells from the graft were not available in our laboratory.

In conclusion, this study shows that in HLA-identical living-related kidney transplant recipients the AZA dose can be safely reduced without the occurrence of acute rejection. Tapering of AZA showed no effect on kidney function, even at 1-year follow-up. No increase in T-cell reactivity directed to donor antigens is found. Therefore, we think that these patients are still over-immunossuppressed, and we suggest to reduce the immunosuppressive medication even further.

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Chapter 9

Successful tapering of immunosuppression to low-dose monotherapy steroids after living-related human leukocyte antigen-identical renal transplantation

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ABSTRACT

Introduction: Living-related (LR) human leukocyte antigen (HLA)-identical renal transplant (RTx) recipients often receive standard immunosuppression, despite the absence of mismatched major HLA-antigens and the known complications of long-term use of immunosuppression. No data are available on the need for immunosuppression for these specific patients. We wondered whether their immunosuppressive load could be radically reduced.

Method: Between November 1982 and November 2005, 83 living-related HLA-identical RTx were performed in our center. Their unadjusted graft survival was 74% at 10 years. In 29 patients (median time after transplantation 5.6 (range 1.0-21.4) years) with stable uncompromised renal function, we tapered their immunosuppression from triple or dual therapy to prednisolone 5 mg/day. Follow up on prednisolone monotherapy was at least 24 months.

Results: In 27 of 29 patients reduction of immunosuppression to prednisolone monotherapy was uneventful. One patient, using dual therapy, developed JC-virus nephropathy resulting in graft loss. One refused further discontinuation of his medication. Four (15%) of the 27 patients on monotherapy developed-biopsy proven recurrence of their original disease. Only one of them showed a transient decline in renal function. One additional patient developed minor proteinuria and a rise in serum creatinine level, as a result of chronic urinary tract infections. The remaining 23 of 27 (85%) patients had an uneventful follow up during 24 months prednisolone monotherapy.

Conclusion: We conclude that HLA-identical LR RTx recipients who are at least 1 year after transplantation might be treated with low-dose steroid monotherapy. Close surveillance of patients for recurrence of their original disease is recommended to allow for potential early therapeutic intervention.

INTRODUCTION

After human leukocyte antigen (HLA)-identical living-related (LR) renal transplantation (RTx) there is less acute rejection and better graft survival compared with nonidentical renal transplant recipients. These superior results are because HLA-identical LR transplants are less immunogenic than nonidentical renal transplants, because in HLA-identical LR RTx all major (class I and II) HLA molecules are identical and only mismatches in minor histocompatibility antigens (mHAgs) or non-HLA antigens may exist. In animal models, the importance of mHAgs has been shown after cardiac transplantation and allogeneic bone marrow transplantation.[1-5] In humans, mismatches in mHAgs have been shown to induce graft-*versus*-host disease after HLA-identical bone marrow transplantation, but minor HLA mismatches had no influence on 5-year graft outcome after RTx.[6, 7]

Nevertheless, recipients of HLA-identical LR donor kidney generally receive the same immunosuppressive regime as HLA-mismatched renal transplant recipients. Therefore, they remain at risk for cardiovascular disease, metabolic complications, infections, and malignancies, all known side effects of immunosuppressive medication and familiar risk factors for poor patient and graft survival after RTx.[8]

Insufficient data are available about immunosuppression after HLA-identical LR RTx.[9-13] Even less data are available of the possibility to reduce or discontinue their immunosuppressive medication.[14] Considering this, we designed a study to reduce the immunosuppressive load dramatically in this specific patient group.

MATERIALS AND METHODS

Patients

Between November 1982 and November 2005, 83 LR HLA-identical RTx were performed in our center. Molecular HLA typing was performed on DNA obtained from blood by polymerase chain reaction (PCR)/sequence-specific oligonucleotide using a reverse dot-blot method.[15] Al study patients who were transplanted before this technique was available were retyped with this PCR technique to be sure they were really HLA-identical with their donor. A transplant was classed as HLA-identical if donor and recipient were reported to have identical HLA A, B, Cw, DR, DP and DQ antigens. All patients had negative cross matches with their donor prior to transplantation. Of these 83 patients, 43 (52%) were men and 40 (48%) were women. Their median age was 50 yrs (range 21-78 years) (Table 1). At time of observation (n=83), the median time after transplantation was 7.0 years (range 0.8-23.8 years). Their unadjusted graft survival was 74% at 10 years, compared to 61% after LR HLA-mismatched renal transplantation (Figure 1).

Out of 83 HLA-identical LR RTx recipients, 54 patients could not be enrolled in our study (demographics see Table 1). Seven (13%) patients died before with a functioning kidney (median time after RTx was 9.6 years, range 0.9-15.6 years), as a result of cardiovascular disease (n=4), infection (n=1), malignancy (n=1), and suicide (n=1).

In 6 (11%) patients graft loss was observed, as a result of recurrence of the original disease (n=1), chronic rejection (n=2), tubular interstitial nephritis due to medication (n=1), surgical complication (n=1), and infection (n=1).

Two (4%) patients were less then 1 year after transplantation, 3 (6%) patients received a kidney from their identical twin sister, 1 (2%) patient already used monotherapy,

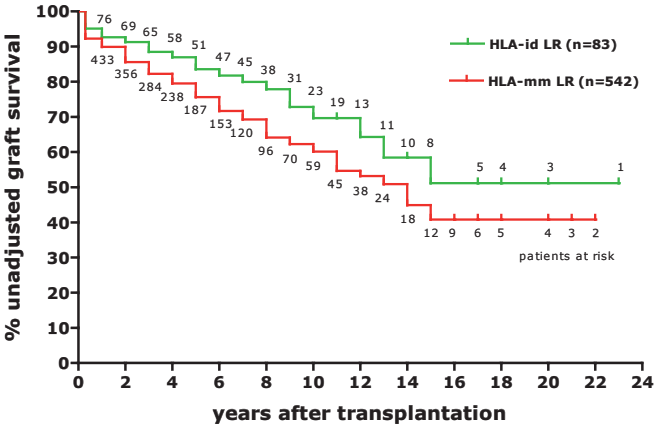

Figure 1: Unadjusted graft survival after living-related HLA-identical (green line) and HLA-mismatched (red line) renal transplantation.

in 1 (2%) patient was transplanted for the fourth time, 11 (20%) patients were excluded for logistic reasons and 7 (13%) refused to participate in the present study.

Seventeen (31%) patients could not be included because of proteinuria. Of these 17 patients, 6 patients had a biopsy-proved recurrence of their original disease, 5 patients had a chronic allograft nephropathy (CAN), in 1 patient the proteinuria disappeared after nephrectomy of his native kidneys and in 5 patients, the cause of their proteinuria remained unknown.

The ethical review committee of our center approved the protocol that was conducted according to local requirements. After informed consent, 29 LR HLA-identical renal transplant recipients who were more then 1 year after RTx, with stable renal function, without proteinuria (<0.2 g/L) and on triple or dual immunosuppression were enrolled in our immunosuppression reduction study. Their demographics are shown in Table 1. The median time after transplantation was 5.6 years. Only 5 patients were more than 10 years after transplantation.

Table 1: Baseline characteristics of the living-related HLA-identical renal transplant recipients transplanted between November 1982 and November 2005.

	Total HLA-identical RTx	Not enrolled	Study Group
Number of patients	83	54	29
Male: female	43:40	28:26	15:14
Age (median in yrs, range)	50 (21-78)	50 (26-78)	51 (21-66)
Male recipient, female donor	28/43 (65%)	18/28 (64%)	10/15 (67%)
Female recipient, male donor	17/40 (43%)	11/26 (42%)	6/14 (43%)
Time after RTx (median in yrs, range)	7.0 (0.8-23.8)	8.8 (0.8-23.8)	5.6 (1.0-21.4)
Original disease with potential to recur	42 (51%)	26 (49%)	16 (55%)

HLA, human leukocyte antigen; RTx, renal transplantation.

Depending on the medication patients used at time of inclusion, we started tapering their calcineurin inhibitor (CNI), followed by mycophenolate mofetil (MMF) or azathioprine (AZA) and prednisolone dose with 2 months regular intervals to prednisolone monotherapy of 5 mg/day. Serum creatinine levels and proteinuria were monitored. A renal biopsy was taken if patients developed a clinically relevant rise of serum creatinine or proteinuria (defined as >0.5 g/L). Blood was obtained for monotoring T-cell reactivity.[16]

Statistical methods

Data for this study were obtained by patient chart analysis. Survival curves were made using the Kaplan-Meier method, and the log-rank test was used to compare the survival rates. Continuous variables are reported as means ± SD and tested by paired Student's *t*-test. Data that did not follow a normal distribution are presented as medians and tested by Wilcoxon signed rank test. Qualitative variables are reported as percentages and were tested by the Pearson's chi-squared test. The SSPS statistical package version 12.0.1 was used. P-values <0.05 were considered significant

RESULTS

Twenty-nine HLA-identical LR RTx recipients were included in our immunosuppression reduction study. In 27 patients this was their first, for 1 patient it was his second, and for 1 it was her third RTx. Their median panel reactive antibody (PRA) was 2% (range, 0-98%) before transplantation.

Most of the patients used dual immunosuppressive therapy at inclusion. Nineteen (66%) used AZA combined with prednisolone, 4 (14%) patients used tacrolimus (Tacro) combined with mycophenolate mofetil (MMF), 3 (10%) patients used MMF combined with prednisolone, and 1 (3%) patient used cyclosporine (CsA) combined with prednisolone. Two patients (7%) used triple immunosuppression consisting of Tacro, MMF and prednisolone. Figure 2 shows the results of serum creatinine levels and proteinuria at time of inclusion

Table 2: Characteristics of patients who developed proteinuria or a rise in their serum creatinine during tapering of their immunosuppressive medication.

Patient	Serum creatinine (μmol/l)	Proteinuria (g/l)	Medication	Years after RTx	Diagnose
7	Stable (102→103)	+ (1.5)	Pred mono for 16.8 months	4.6	Recurrence membranous nephropathy
11	↑ (108→126)	+ (0.74)	Pred mono for 2 months	3.3	Chronic urinary tract infection
13	↑ (87→213)	- (0.02)	MMF 500 mg + Pred 5 mg	1.1	JC-virus infection
24	Stable (58→64)	+ (1.73)	Pred mono for 21.7 months	7.0	Recurrence membranous nephropathy
21	Stable (133→107)	+ (2.96)	Pred mono for 2.1 months	10.7	Recurrence diabetes nephropathy
29	↑ (111→203)	- (0.09)	Pred mono for 13.3 months	7.8	Recurrence IgA nephropathy

Clinical relevant proteinuria is defined as > 0.5 g/L. "Medication" is defined as the medication patients used at the moment their serum creatinine rise and/or they developed proteinuria. Diagnose is made on clinical grounds and confirmed by renal biopsy. Pred, prednisolone; MMF, mycophenolate mofetil; RTx, renal transplantation.

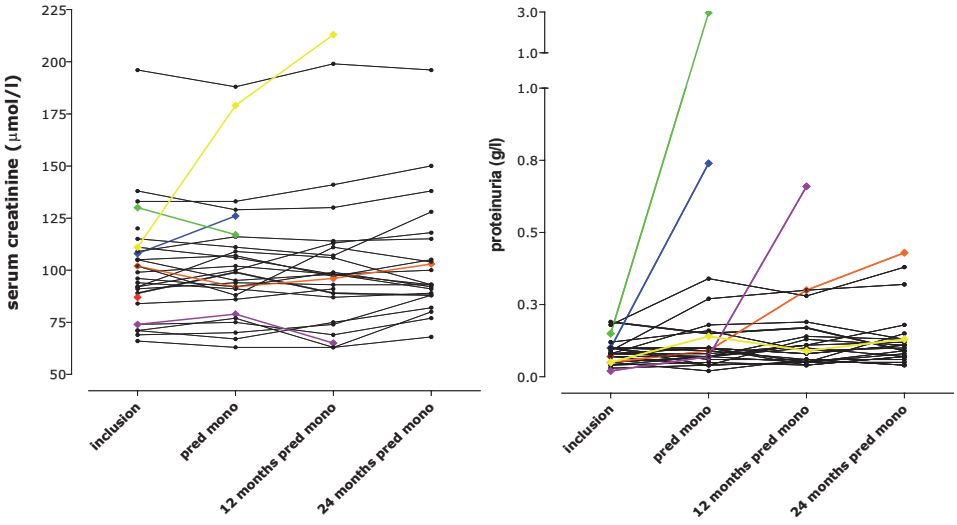

Figure 2: Serum creatinine levels and proteinuria at time of inclusion (using different combinations of immunosuppressive medication), at the moment patients started with prednisolone monotherapy 5 mg/day and after they had been on prednisolone monotherapy for 12 and 24 months.

(using different combinations of immunosuppressive medication), at the moment patients started with prednisolone monotherapy 5 mg/day and after they had been on prednisolone monotherapy for 12 and 24 months.

One patient refused further discontinuation of his immunosuppressive medication after inclusion in the study. Another patient, still on dual therapy, developed a biopsy proved JC-virus nephropathy 13.3 months after transplantation. Despite reducing her immunosuppressive medication to prednisolone 10 mg/day, combined with leflunomide 30 mg/day, she had a progressive decline of her creatinine clearance resulting in graft loss 25.3 months after RTx.

Four (15%) of the 27 patients showed a recurrence of their original disease in their renal biopsy, after being on monotherapy prednisolone 5 mg/day for 2, 13, 17 and 22 months and 11, 8, 5 and 7 years after RTx, respectively. Despite proteinuria, serum creatinine levels remained stable in three of them. The other showed a transient rise in serum creatinine level, due to IgA nephropathy, which stabilized after reintroduction of MMF 2 g/day and raising the prednisolone dose to 10 mg/day. Another patient showed a rise in serum creatinine level because of a chronic urinary tract infection after several urological procedures (Table 2).

In 23 (85%) of the 27 patients, the immunosuppression could be successfully reduced to prednisolone monotherapy 5 mg/day. No significant changes in serum creatinine levels, 99 µmol/L (range 66-196) vs. 93 µmol/L (range 68-196), or protein excretion, 0.08 (range 0.02-0.19) vs. 0.10 (range 0.04-0.38) g/L, were observed between time of inclusion and after 24 months of prednisolone monotherapy 5 mg/day. There were no significant changes in systolic-, diastolic blood pressure, the number of antihypertensive drugs taken,

serum total-, low-density lipoprotein (LDL)- or high-denstity lipoprotein (HDL) cholesterol levels, haemoglobulin, thrombocytes, or leucocytes between time of inclusion and after 24 months of prednisolone monotherapy.

DISCUSSION

With the current results of patient and graft survival after RTx, we are confronted with the inherited complications of long-term use of immunosuppressive medication. Therefore, we have the obligation to investigate the possibilities of tapering this medication without reducing the short- and long-term graft and patient survival. A lot of our study patients were treated with azathioprine and prednisolone, by many classified as "light immunosuppression". These patients were probably at low risk for rejection after tapering immunosuppression and this is exactly what we showed. Moreover, we should keep in mind that even maintenance therapy with so-called "light immunosuppression" is accompanied with serious side effects.

In our opinion, in LR HLA-identical RTx recipients monotherapy with low-dose steroids, although not devoid from all side effects, is preferred above monotherapy with low-dose AZA, MMF or CNI, with regard to infections and malignancies. This also holds true for recently described protocols with antithymocyte globulin, total lymphoid irradiation, and hematopoietic-cell transplantation.[17] In our LR HLA-identical RTx study group, dramatically tapering of their immunosuppressive medication to low dose prednisolone monotherapy is well tolerated, without the occurrence of acute rejections during a follow up of 2 years. Acute rejection episodes after identical sibling RTx have been reported. However, most of them were described in the AZA era in a time class II match was not perfect and BK-virus nephropathy was an unrecognized entity. Nevertheless, mismatches in minor HLA-antigens has been found relevant in the context of bone marrow transplantation and might theoretical induce immunological reactivity against minor mismatched solid organs. Recently, Gerrits *et al.* described in vitro reactivity against donor cells after HLA-identical LR RTx, but could not prove that this was the result of mismatches in minor HLA-antigens.[18] Thus, acute rejection after tapering immunosuppression could be immunologically explained. However, it did not occur in our study, which is in line with the observation of Heinold *et al*., who did not find a clinical relevant role of minor HLA mismatches after solid organ transplantation.[7] Differences in non-HLA antigens could be an alternative explanation for donor reactivity after HLA-identical RTx.[19, 20]

After tapering their immunosuppressive medication, recurrence of original disease occurred in 15% (4 of 27) of the patients of the total study group, or otherwise specified, in 25% (4 of 16) of the group of patients who had an original disease with potential to recur. After 2 years follow up, none of their renal grafts had failed. It should be mentioned that one of these four patients had a diabetes nephropathy. We wondered whether the recurrence of primary glomerulonefritis in the other three patients could be related to the tapering of their immunosuppressive load or that this just reflects the natural course of recurrence of a primary glomerulonephritis after RTx. In 1999, Andresdottir *et al.*[21] described a biopsy proven prevalence of recurrence of original disease after LR HLA-identical RTx of at least 27%, with a graft failure due to recurrence of 15%, with a mean time after transplantation of 7.7 ±6.1 years.

Before we embarked on the present study, we screened our LR HLA-identical population under full dose immunosuppression. There was a prevalence of 17% (7 out of 42) of biopsy-proven recurrence of original disease, in the group of patients who had that

potential. In five patients with proteinuria, no histology was available, so the true incidence of recurrence could have been as high as 35%. This suggest that the prevalence of recurrence after tapering immunosuppressive medication in our study group was comparable to that described before in LR HLA-identical RTx recipients who used full-dose immunosuppression and is in line with earlier observation.

In conclusion, the immunosuppressive medication can be safely reduced to low-dose steroid monotherapy of 5 mg/day in HLA-identical LR renal transplant recipients provided that they have stable renal function, without proteinuria and they are at least 1 year after transplantation. Close surveillance of patients for recurrence of their original disease is recommended to allow for potential early therapeutic intervention.

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Chapter 10

T-cell reactivity during tapering of immunosuppression to low-dose monotherapy prednisolone in HLA-identical living-related renal transplant recipients

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ABSTRACT

Background: In many transplant centers, human leukocyte antigen (HLA)-identical living-related (LR) renal transplant recipients receive standard maintenance immunosuppression from 1 year after transplantation. We questioned whether discontinuation of azathioprine (AZA) or mycophenolate mofetil (MMF) influenced T-cell reactivity, circulating DC subsets numbers, and their maturation status.

Methods: Twenty-nine HLA-identical LR renal transplant recipients were withdrawn from AZA or MMF. Thereafter, the patients received only prednisolone. T-cell reactivity was determined by interferon (IFN)-γ (n=23), interleukin (IL)-10 (n=16), and granzyme B (GrB; n=10) Elispot assays. Circulating DC subset numbers and their maturation status determined by CCR2, CCR5, CCR7 and CD83 expression were measured by flow cytometry (n=12).

Results: The number of donor, 3rd-party, and tetanus toxoid (TET)-reactive IFN-γ and GrB producing cells (pc) was not affected after withdrawal of immunosuppression. Discontinuation of AZA or MMF resulted in significant increased numbers of 3rd-party (p=0.003) and TET-reactive (p=0.008) IL-10 pc, and a trend in higher numbers of donor-reactive IL-10 pc (p=0.06). No effect was found on the number of circulating DC subsets, but DC were shifted towards a more mature phenotype.

Conclusions: In HLA-identical LR renal transplant recipients, therapy with AZA and MMF suppress the IL-10 production and the maturation of DC. This suggests that these immunosuppressants may hinder downregulation of immune responses in general, including allogeneic responses.

INTRODUCTION

After human leukocyte antigen (HLA)-identical LR renal transplantation, mismatches only in minor histocompatibility antigens (mHAgs) and other non-HLA antigens may exist between donor and recipient.[1, 2] mHAgs are genetic inherited peptides derived from polymorphic intracellular proteins presented in the context of HLA class I and II molecules and can be recognized by T cells. In humans, the clinical relevance of mHAgs has been reported after bone marrow transplantation[2], and even after solid organ transplantation a role of mHAgs and non-HLA antigens has been suggested.[3-5] Theoretically, both mismatches in mHAgs and other non-HLA antigens between donor and recipient might induce graft rejection. Consequently, HLA-identical LR renal transplant recipients still receive immunosuppression. However, the necessity for long-term use of immunosuppression in this patient group is yet unknown. We wondered whether HLA-identical LR renal transplant recipients should be exposed to the severe side effects of immunosuppression such as nephrotoxicity, malignancies, cardiovascular disease and diabetes mellitus.[6, 7]

Previously, we demonstrated that donor-reactive cytokine responses can be found after HLA-identical LR renal transplantation.[8] Additionally, we showed that HLA-identical LR renal transplant recipients, who received azathioprine (AZA) in combination with prednisone, could be tapered to 50% of their original AZA dose without the occurrence of acute rejection. Furthermore, renal function and donor-reactive responses remained unaffected after tapering the AZA dose.[9, 10] Therefore, we speculate that those patients are still over-immunosuppressed and that the immunosuppressive dose could be reduced further.

Increased donor reactivity could occur after discontinuation of immunosuppression in transplant recipients after HLA-mismatched renal transplantation.[11-14] Complete discontinuation of immunosuppression has been reported in a minority of renal transplant recipients long after transplantation with stable graft function and without clinical signs of rejection.[15-20] In general, a decreased T-cell response was reported in those studies compared to patients with chronic allograft nephropathy.[15, 16, 19, 20]

In addition to immunological monitoring of donor-reactive T-cell responses in transplant recipients, it has been suggested that monitoring of circulating myeloid dendritic cells ($CD11c^{+}CD123^{low}BDCA\text{-}1^{+}$ mDC) and plasmacytoid DC ($CD11c^{-}CD123^{high}BDCA\text{-}2^{+}$ pDC)[21] numbers in peripheral blood might be an useful tool for identifying transplant recipients in whom the immunosuppressive load can be safely tapered.[22, 23] mDC produce high levels of interleukin (IL)-12 and induce T-helper 1 (Th1) and cytotoxic T-cell (CTL) responses, whereas pDC produce interferon (IFN)-α in response to viruses and induce T-helper 2 (Th2) responses.[24] Furthermore, it has been suggested that pDC are involved in the induction of peripheral T-cell tolerance after organ transplantation.[23, 25] According to their surface immunophenotype, DC subsets can be identified as immature DC and mature DC.[24, 26] In peripheral blood and tissues, DC resides as immature DC where they may internalize antigens. On antigen capture, immature DC differentiates into mature DC that are highly specialised to stimulate T cells efficiently.[27] Several studies reported the influence of immunosuppressive drugs on DC subset numbers, differentiation, and their maturation status.[23, 28-33] Furthermore, Mazariegos *et al.*[23] reported that the proportion of pDC in peripheral blood mononuclear cells (PBMC) was higher in stable liver transplant recipients who could be successfully weaned from their immunosuppressive load.

In the present study, we discontinued the AZA and mycophenolate mofetil (MMF) dose. Thereafter, all patients received steroid monotherapy for at least 1 year. We questioned whether discontinuation of AZA or MMF influenced T-cell reactivity determined by Elispot

assays. This assay was used to determine the frequency of pro-inflammatory cytokine IFN-γ and anti-inflammatory cytokine IL-10 that have been associated with allograft rejection or suppression of the immune response, respectively.[34, 35] Granzyme B (GrB) was used as a marker of activity of cytotoxic T-lymphocytes (CTL).[10, 36] CTL plays a crucial role in allograft rejection.[37] Additionally, we wondered whether discontinuation of immunosuppression affected the circulating DC subsets numbers and their maturation status determined by flow cytometry.

MATERIALS AND METHODS

HLA-identical LR renal transplant recipients

The ethical review committee of our center approved the protocol that was conducted according to local requirements. Between November 1982 and November 2005, 83 LR HLA-identical renal transplants were performed in our center. Out of those 83 patients, 54 patients could not be enrolled in our study: seven patients died with a functioning kidney (n=4: cardio-vascular disease; n=1: infection; n=1: malignancy; n=1: suicide), in six patients graft loss was observed (n=1: recurrence of original disease; n=2: chronic rejection; n=1: tubular intestinal nephritis due to medication; n=1: surgical complication; n=1: infection), two patients were less than 1 year after transplantation, three patients received a kidney from their HLA-identical twin sister, one patient already used monotherapy, one patient was transplanted for the fourth time, 11 patients were excluded for logistic reasons, seven patients refused to participate in the present study, and 17 patients could not be included because of proteinuria (n=6: recurrence of their original disease; n=5: chronic allograft nephropathy; n=1 proteinuria disappeared after nephrectomy of his native kidneys, n=5: cause of proteinuria is unknown).[38] After informed consent, 29 HLA-identical LR renal transplant recipients agreed to participate in this study. Characteristics of the patients are described in Table 1. The patients had stable serum creatinine levels and no proteinuria (<0.5 g/L) were more than 1 year after transplantation, and on triple or dual immunosuppressive therapy. From the 29 patients, 19 patients used AZA in combination with prednisolone and three patients used MMF in combination with prednisolone. The other seven patients (n=4, Tacro+MMF; n=1, CsA+prednisolone; n=2, Tacro+MMF+prednisolone) were converted to MMF (500 mg/day) in combination with prednisolone (5 mg/day). Then, the AZA or MMF dose was gradually discontinued over a period of 4 months and patients were kept on prednisolone (5 mg/day) monotherapy. The follow-up of the patients on prednisolone monotherapy was 1 year (Figure 1). Our laboratory analysis on T-cell reactivity started at dual therapy and 1 year monotherapy (Figure 1).

Blood sampling

We received 35 ml heparinized peripheral blood at dual therapy and monotherapy to perform Elispot assays (33 ml peripheral blood), and to measure DC subset numbers and their maturation status (2 ml peripheral blood). PBMC from recipient and donor were isolated from heparinized blood by density gradient centrifugation using Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) and stored at −140°C as described before.[9]

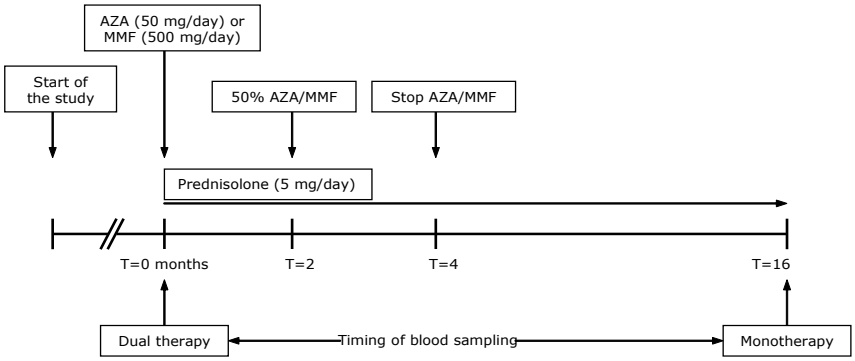

Figure 1: Schematic overview of discontinuation of immunosuppressive medication in HLA-identical living-related renal transplant recipients and time of blood sampling (dual therapy and 1-year steroid monotherapy).

IFN-γ, IL-10, and GrB Elispot assays

The phytohemagglutinin (PHA; Murex Biotech, Kent, UK) proliferation assay was performed to control the viability of the PBMC as described before.[9] The mean counts per minute (cpm) were determined, and the stimulation index (SI) was calculated by the ratio of the cpm obtained in the presence of PHA to the cpm in the absence of PHA. Only results of viable cells (SI ≥ 50) were analyzed in the described results.

The IFN-γ, IL-10, and GrB Elispot assays (U-CyTech Biosciences, Utrecht, The Netherlands) were used to determine the number of cytokine producing cells (pc) reactive to donor, 3rd-party, and tetanus toxoid (TET) antigens.[39] Briefly, in a 96-wells round bottom plate (Nunc, Roskilde, Denmark), patients' PBMC were stimulated with donor PBMC, 3rd-party PBMC, and TET. The 3rd-party PBMC did not share HLA antigens with donor and patient, and the same 3rd-party PBMC was used at dual therapy and steroid monotherapy. TET stimulation (RIVM, Bilthoven, The Netherlands) was used to determine the memory immune response to nominal antigens. As negative controls, we used patients' PBMC stimulated with irradiated patients' PBMC (autologous response; to control the influence of irradiation), and patients' PBMC in culture media alone (unstimulated patients' PBMC). The autologous response was subtracted from the donor and 3rd-party reactive response. The response of unstimulated patients' PBMC was subtracted from the TET response. After 40 hr of incubation, non-adherent cells were harvested and transferred in triplicate to a flat-bottom plate (Nunc, Roskilde, Denmark) precoated with IFN-γ, IL-10, or GrB monoclonal antibodies (U-CyTech Biosciences) and post-coated with phosphate-buffered saline (PBS) supplemented with 1x Blocking stock solution B (U-CyTech Biosciences). Cells were incubated for 5 hr at 37°C for IFN-γ and GrB Elispot assays, and overnight for IL-10 Elispot assay. Detection of spots was performed as described before.[39] The spots were counted automatically by using a Bioreader 3000 Elispot reader (BioSys, GmbH, Karben, Germany).

Antibodies for mDC and pDC staining

Fluorescence-activated cell sorter (FACS) analysis was performed using the following mouse anti-human monoclonal antibodies: allophycocyanin (APC)-conjugated BDCA-1 (clone: AD5-

Table 1: Characteristics of the HLA-identical living-related renal transplant recipients

Patient	Gender		Primary disease	T_{DT}[3] (years)	# RTx[4]	IS[5] (+prednisolone 5 mg/day)	Reached monotherapy?	Tested in Elispot assays?	Analysed by flow cytometry?
	[1]P	[2]D							
1	M	M	Focal segmental glomerulosclerosis	1.6	1	MMF[8]	no	-[10]	-
2	F[6]	M[7]	AL-amyloïdose	1.7	1	AZA[9]	yes	+[11]	+
3	F	F	Rapidly progressive glomerulonephritis	4.4	1	AZA	yes	+	-
4	F	F	Focal segmental glomerulosclerosis	12.3	1	AZA	yes	+	-
5	M	M	Focal segmental glomerulosclerosis	4.9	1	MMF	yes	+	+
6	M	M	Membranous glomerulonephritis	7.1	1	AZA	yes	+	+
7	M	F	Membranous glomerulonephritis	3.0	1	MMF	yes	+	+
8	M	F	Hypertension	13.6	1	AZA	yes	+	+
9	F	M	Chronic pyelonephritis	22.0	1	MMF	yes	+	+
10	F	F	Medullary cystic disease	2.9	1	AZA	yes	+	+
11	F	F	Congenital obstructive nephropathy	3.9	1	MMF	yes	-	-
12	M	F	Acute tubular necrosis	6.8	1	AZA	yes	+	-
13	F	M	Polycystic kidney disease	1.0	1	MMF	no	-	-
14	M	F	Membranous glomerulonephritis	11.2	1	AZA	yes	+	+
15	F	F	Unknown	17.3	1	AZA	yes	+	-
16	M	M	IgA nephropathy	6.7	1	AZA	yes	+	-
17	M	F	Hypertension	2.5	1	MMF	yes	+	+
18	F	M	SLE nephropathy	9.0	1	AZA	yes	+	-
19	M	F	Von Hippel Lindau	6.3	1	AZA	yes	+	+
20	F	F	IgA nephropathy	5.4	3	MMF	yes	+	-
21	M	F	Diabetes nephropathy	10.5	1	MMF	yes	-	-
22	M	F	Necrotic glomerulonephritis eci	2.3	1	MMF	yes	+	+
23	F	F	Meningococcal sepsis	4.4	1	AZA	yes	+	-
24	F	M	Extracapillary glomerulonephritis	5.1	1	AZA	yes	+	-
25	F	F	Chronic pyelonephritis	12.5	1	AZA	yes	+	-
26	M	M	Adult polycystic kidney disease	7.2	1	AZA	yes	-	-
27	M	F	Reflux nephropathy	5.4	2	AZA	yes	+	-
28	F	M	Diabetes Mellitus II and hypertension	5.6	1	AZA	yes	+	+
29	M	F	IgA nephropathy	6.6	1	AZA	yes	-	-
			median	6.3 (1.0-22.0)	1				

HLA, Human leukocyte antigen; P, patient; D; donor; TDT, time from transplantation to inclusion of study (dual therapy: DT); RTx, first, second or third renal transplantation, IS, immunosuppressive medication at dual therapy; F, female; M, male; MMF, mycophenolate mofetil; AZA, azathioprine; -, not determined; +, determined.

8E7) and APC-conjugated BDCA-2 (clone: AC144) (Miltenyi Biotec, GmbH, Germany), peridinin chlorophyll protein (PerCP)-conjugated CD14 (clone: mφP9) and PerCP-conjugated CD19 (clone: 4G7; Becton Dickinson Biosciences, San Jose, CA, USA), fluorescein isothiocyanate (FITC)-conjugated CD83 (clone: HB15A17.11; DPC, Serotec, Oxford, UK), phycoerythrin (PE)-conjugated CCR2 (clone: 48607.211) and PE-conjugated CCR7 (clone: 150503; R&D Systems Europe, Abingdon, UK), FITC-conjugated CCR5 (clone: 2d7; Becton Dickinson), and FITC-conjugated IgG2a (clone: X39) and PE-conjugated IgG2b (clone: X39) isotype control monoclonal antibodies (Becton Dickinson).

Immunofluorescence staining and flow cytometric analysis of dendritic cell subsets and their maturation status

Analysis of DC numbers and maturation status was performed as described before.[40] Briefly, 2 ml fresh heparinized blood was obtained from 12 HLA-identical LR renal transplant recipients (patient: 2, 5, 6, 7, 8, 9, 10, 14, 17, 19, 22, 28; Table 1) and processed within 4 hr. Whole blood samples were incubated with the abovementioned monoclonal antibodies for 30 min in the dark at room temperature. Cells that stained negative for CD14 and CD19 were gated and analyzed for BDCA-1 and BDCA-2 expression. mDC and pDC were identified as $CD14^{-}CD19^{-}$ $BDCA\text{-}1^{+}$ cells and $CD14^{-}CD19^{-}BDCA\text{-}2^{+}$ cells, respectively. Immature mDC were defined as $CD83^{-}CCR7^{-}CCR5^{+}CCR2^{+}$, and mature mDC as $CD83^{+}CCR7^{+}CCR5^{-}CCR2^{-}$. Immature pDC were defined as $CD83^{-}CCR7^{+}CCR5^{+}CCR2^{+}$, and mature pDC as $CD83^{+}CCR7^{++}CCR5^{-}CCR2^{-}$. The proportion of mDC and pDC positive for CD83, CCR7, CCR5, and CCR2 was determined by comparison to their respective isotype control antibodies. From each tube, 500,000 events were acquired on a FACScalibur flow cytometer using CELLQUEST PRO software (Becton Dickinson).

The absolute counts for each DC subset was calculated by multiplying the proportion of mDC and pDC within the total leukocyte population by the absolute number of white blood cells determined on an automatically cell counter (Casey®, Schärfe System, GmbH, Rentlingen, Germany). The absolute counts for total DC was calculated by the sum of the absolute counts of mDC and pDC. The pDC/mDC ratio was determined by dividing the absolute number of pDC with the absolute number of mDC.

Statistical analysis

The Wilcoxon signed rank test was used to compare the frequency of cytokine pc, the absolute number of total DC (mDC+pDC), mDC and pDC numbers, and the pDC/mDC ratio at dual therapy and monotherapy. The same test was used to compare the percentage positive of CD83, CCR7, CCR2 and CCR5 on DC subsets before and after discontinuation of AZA or MMF. The Fischer's Exact test was used to compare the number of patients who responded to TET antigens before and after withdrawal of AZA or MMF. Two sided P-values ≤ 0.05 were considered significant. For statistical analysis, SPSS 11.5 for Windows was used (SPSS, Inc., Chicago, IL, USA).

RESULTS

Clinical results

After inclusion (dual therapy), 2 of 29 (7%) HLA-identical LR renal transplant recipients did not reach steroid monotherapy because to JC-virus infection (patient 13) and one patient (patient 1) refused to discontinue his immunosuppressive medication after inclusion in the study (Table 1). None of the patients had an acute rejection episode. A detailed description of the clinical results of this study is described by Van de Wetering *et al.*[38].

Patient 11, 21, 26 and 29 (n=4) were not tested in the cellular assays, because we were unable to receive patient and donor PBMC, respectively (Table 1).

We received 35 ml heparinized blood during dual therapy and during monotherapy. The PBMC yield is variable after transplantation. From all patients described below, PBMC samples were tested during dual and after 1 year on monotherapy. We could perform an IFN-γ Elispot assay in 23 patients, from 16 patients enough cells were available to perform the IL-10 Elispot, and even from 10 patients we also could perform a GrB Elispot.

In 2003, it was reported that monitoring of circulating DC would be a good tool to identify transplant recipients in whom the immunosuppressive medication can be safely discontinued.[23] Then, we observed that cryopreservation of PBMC significantly effects chemokine receptor markers on DC.[40] In other words, we could only determine the DC subsets and their chemokine receptors in fresh whole blood. Our inclusion of patients was from the beginning of 2003. Therefore, we have monitored the DC in 12 patients before and after withdrawal of AZA or MMF.

The frequency of IFN-γ, IL-10 and GrB producing cells

The frequency of donor-reactive IFN-γ pc did not increase after discontinuation of AZA or MMF (dual therapy: median, 2 IFN-γ pc/2x10^5 PBMC [range, 0-13]; monotherapy: median, 0 IFN-γ pc/2x10^5 PBMC [0-16]; p=0.21; Figure 2A). The donor response was significantly lower than the 3rd-party response (dual therapy, p<0.001; monotherapy, p<0.001). The 3rd-party reactivity was comparable between dual therapy (median, 33 IFN-γ pc/2x10^5 PBMC [5-333]) and monotherapy (median, 31 IFN-γ pc/2x10^5 [4-322]; p=0.59; Figure 2B).

From 16 patients, we were able to perform IL-10 Elispot assays. The number of donor-reactive IL-10 pc was low on dual therapy (median, 1 IL-10 pc/2x10^5 PBMC [range, 0-4]) and tended to be higher after discontinuation of AZA or MMF (median, 1 IL-10 pc/2x10^5 [0-80]; p=0.06; Figure 2C). No significant difference was found in donor and 3rd-party reactive IL-10 pc (dual therapy, p=0.18; monotherapy, p=0.06). The frequency of 3rd-party reactive IL-10 pc was significantly higher during monotherapy (median, 13 IL-10 pc/2x10^5 PBMC [0-208]; p=0.003; Figure 2D) than during dual therapy (median, 2 IL-10 pc/2x10^5 PBMC [0-8]).

We were able to perform GrB Elispot assays from 10 patients. No difference was observed between the number of donor-reactive GrB pc at dual therapy (median, 2 GrB pc/2x10^5 PBMC [range, 0-40]) and at monotherapy (median, 0 GrB pc/2x10^5 [0-6]; p=0.18; Figure 2E). The donor-reactive GrB pc was significantly lower than the 3rd-party reactive GrB pc (dual therapy, p=0.008; monotherapy, p=0.007). At dual therapy, the frequency of 3rd-party reactive GrB pc (median, 17 GrB pc/2x10^5 PBMC [0-49]) was comparable with monotherapy (median, 16 GrB pc/2x10^5 PBMC [0-47]; p=0.84; Figure 2F).

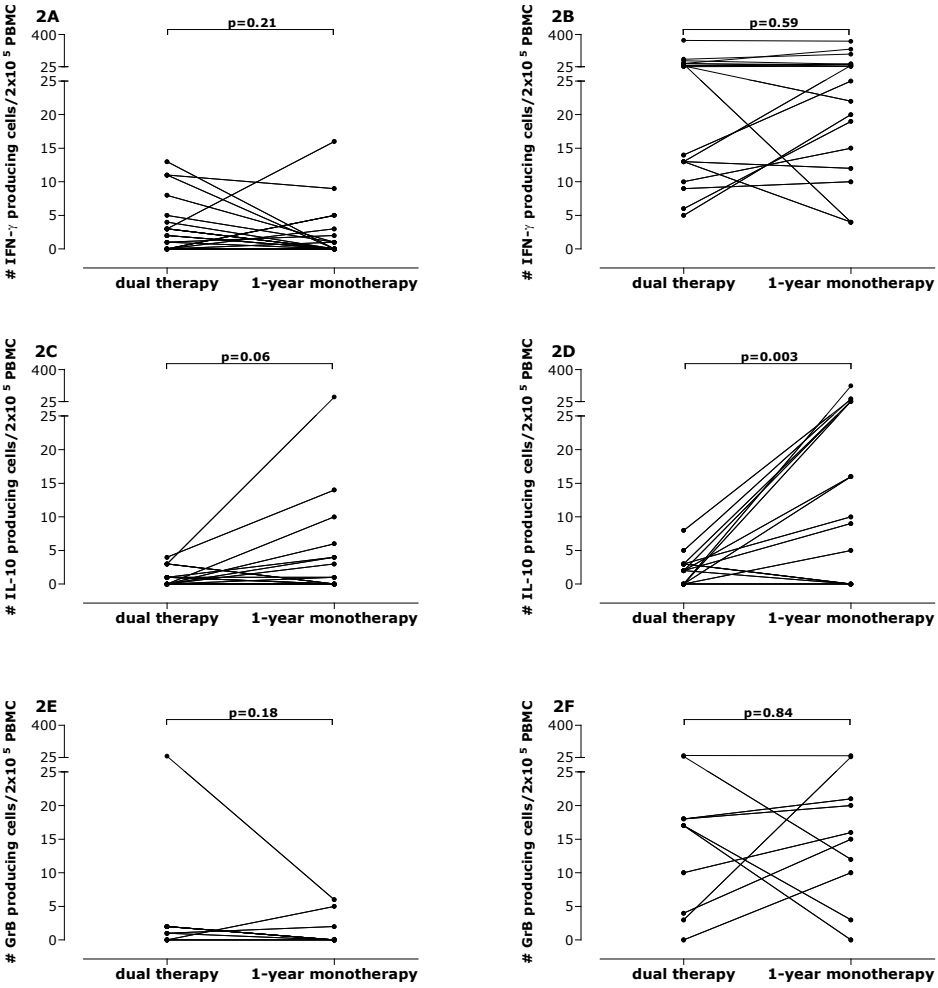

Figure 2: Number of IFN-γ, IL-10 and granzyme B (GrB) pc reactive to donor cells (A, C, E) and 3rd-party cells (B, D, F) before (dual therapy) and after (1-year monotherapy) discontinuation of AZA or MMF in PBMC from HLA-identical living-related renal transplants determined by IFN-γ, IL-10 and GrB Elispot assays.

Tetanus toxoid reactivity

The TET reactivity was not affected after withdrawal of AZA or MMF in IFN-γ Elispot assays (dual therapy: median, 6 IFN-γ pc/2x10^5 PBMC [0-217] vs. monotherapy: median, 4 IFN-γ pc/2x10^5 PBMC [0-179]; p=0.74), and GrB Elispot assays (dual therapy: median, 5 GrB pc/2x10^5 PBMC [0-112] vs. monotherapy: median, 8 GrB pc/2x10^5 PBMC [0-115]; p=0.44).

The total number of TET-reactive IL-10 pc was comparable during dual and monotherapy (dual therapy: median, 3 IL-10 pc/2x10^5 PBMC [0-77] vs. monotherapy: median, 10 IL-10 pc/2x10^5 PBMC [0-102]; p=0.32). However, the number of patients that could respond to TET antigens (≥5 cytokine pc/2x10^5 PBMC) in the IL-10 Elispot assay was significantly higher at monotherapy than at dual therapy (dual therapy, 5/20 [25%] vs. monotherapy, 13/18 [72%]; p=0.008; Fisher's Exact test). No differences were found in IFN-γ (p=0.57) and GrB (p=0.43) Elispot assays.

Dendritic cell subsets and maturation status

Discontinuation of AZA or MMF had no effect on the total DC numbers (dual therapy: median, 14.6 DC/μl [range, 5.5-31.1]; monotherapy: median, 14.4 DC/μl [3.5-26.2]; p=0.52), the number of mDC (dual therapy: median, 10.7 μl mDC [4.0-23.7]; monotherapy: median, 10.0 μl mDC [2.6-20.8]; p=0.62, Figure 3A) and pDC (dual therapy: median, 4.7 μl pDC [1.6-8.8]; monotherapy; median, 3.0 μl pDC [0.93-11.3]; p=0.62, Figure 3E), nor on the pDC/mDC ratio (dual therapy: median, 0.37 [0.23-0.82]; monotherapy: median, 0.33 [0.16-0.96]; p=0.49).

The percentage of CD83$^+$ mDC was not affected after discontinuation of AZA or MMF (dual therapy: median, 0.49% [0-2.8]; monotherapy: median: 0.65% [0.08-1.45]; p=0.47). During monotherapy, the percentage of CCR7$^+$ mDC (median, 22.3% [10.2-28.6]; p=0.007) was significantly higher than during dual therapy (median, 10.3% [5.3-20.9]; Figure 3B). During monotherapy, the percentage CCR2$^+$ mDC (dual therapy: median, 87.6% [49.5-95.9]; monotherapy: median, 69.5% [50.3-91.4]; p=0.01; Figure 3C) and CCR5$^+$ mDC (dual therapy: median, 91.2% [56.1-97.3]; monotherapy: median, 80.1% [53.4-94.6]; p=0.04; Figure 3D) was significantly lower than during dual therapy.

A similar pattern was found for pDC. No differences were observed in the percentage CD83$^+$ pDC (dual therapy: median, 0% [0-0.6]; monotherapy: median, 0% [0-0.9]; p=0.31) during dual and monotherapy. The percentage CCR7$^+$ pDC increased after discontinuation of AZA or MMF [dual therapy: median, 86.8% (81.3-94.7); monotherapy: median, 91.1% (86.6-96.9); p=0.12; Figure 3F]. The percentage CCR2$^+$ pDC (dual therapy: median, 96.8% [92.5-98.5]; monotherapy: median, 94.2% [90.4-98.2]; p=0.04; Figure 3G) and CCR5$^+$ pDC (dual therapy: median, 99.4% [98.3-100]; monotherapy: median, 98.9% [95.9-100]; p=0.02; Figure 3H) decreased after discontinuation of AZA or MMF.

DISCUSSION

After solid organ transplantation, life-long use of immunosuppression is deemed necessary to prevent graft rejection. However, after HLA-identical LR renal transplantation, the necessity for long-term use of immunosuppression is yet unknown. Considering the severe side effects of immunosuppression, minimizing of immunosuppression in these transplant recipients might be beneficial. Theoretically, discontinuation of immunosuppression in organ transplant recipients might result into an increased donor-reactive T-cell response.[12-14, 41] This study showed that the number of donor, 3rd-party, and TET-reactive IFN-γ pc or GrB pc did not increase after discontinuation of AZA or MMF. However, significantly increased numbers of 3rd-party (p=0.003) and TET-reactive (p=0.008) IL-10 pc and a trend in more donor-reactive IL-10 pc (p=0.06) were found in HLA-identical LR renal transplant recipients. Additionally, no acute rejections occurred.[38] We suggest that the suppressive function of IL-10 was hindered

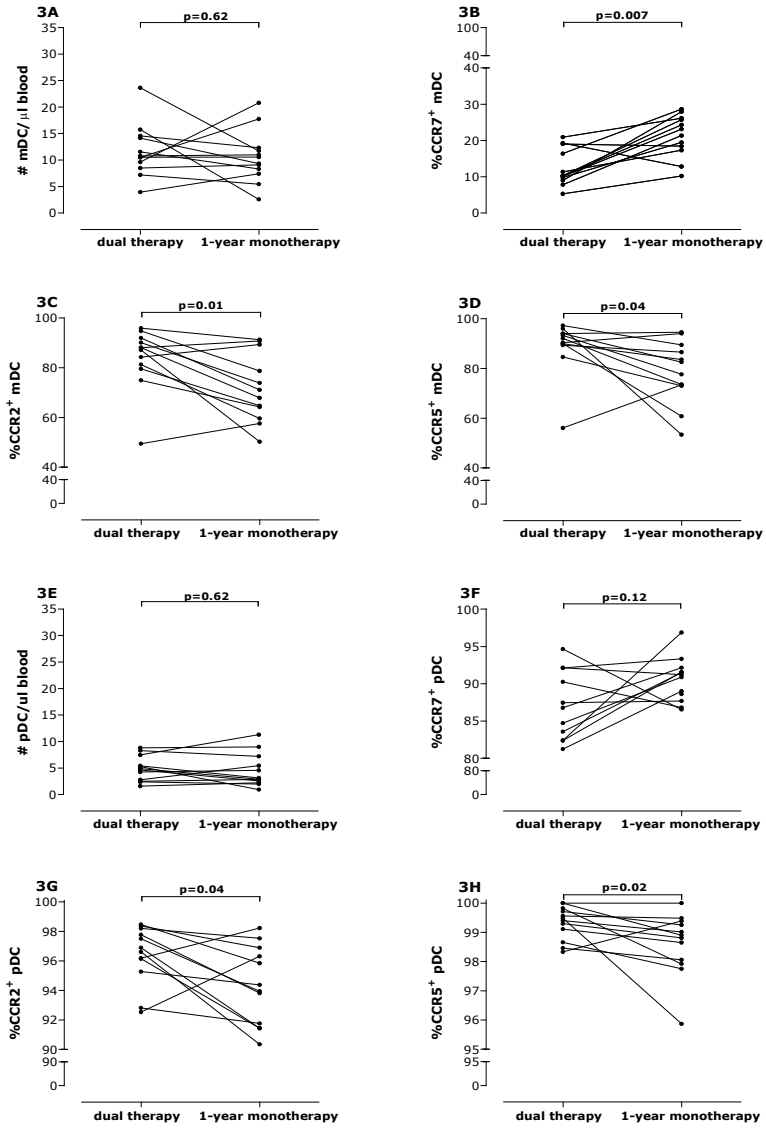

Figure 3: Absolute numbers and their maturation status of mDC (A-D) and pDC (E-H) determined before (dual therapy) and after (1-year monotherapy) discontinuation of AZA or MMF in peripheral blood from recipients of an HLA-identical living-related renal transplant (n=12) by flow cytometry.

by AZA and MMF. AZA and MMF are anti-proliferative agents, and may also have an effect on cytokine production of lymphocytes.[42-44]

IL-10 is an anti-inflammatory cytokine that have been associated with downregulation of the immune response.[45] Several cells can secrete IL-10, such as B cells, monocytes, DC, activated Th2 cells, and regulatory T cells.[45] In our assay, we assume that monocytes and

DC did not produce IL-10, because only non-adherent cells were transferred to the IL-10 Elispot plate. On activation by donor cells, both Th2 cells and regulatory T cells mainly produce IL-10.[46] In the present study, we observed higher numbers of donor, 3rd-party, and TET reactive IL-10 pc after withdrawal of AZA or MMF, while the number of IFN-γ and GrB pc remained stable. In agreement with our results, other studies also reported the presence of donor-reactive IL-10 that could reflect allograft tolerance in HLA-identical LR renal transplant recipients.[20, 47]

Renal transplant recipients who receive long-term immunosuppression are susceptible for infections.[6] Therefore, tapering of immunosuppression may reduce the chance for infections in those patients. In our study, we observed that more patients could respond to TET antigens in IL-10 Elispot assays after discontinuation of AZA and MMF, suggesting an improvement of reactivity directed to nominal antigens.

Mismatches in mHAgs between donor and recipient in combination with the presence of the correct HLA-restriction molecule may trigger T-cell reactivity.[2] We analysed whether donor-reactive T-cell responses could be a result of known mHAg disparities between donor and recipient. In the present study, we again found no relation between mHAg mismatches and the number of donor-reactive cytokine pc, which is in agreement with our previous studies.[9, 39] Furthermore, Heinold *et al.*[5] showed after cadaveric and LR renal transplantation that mHAgs mismatches between donor and recipients had no significant effect on death-censored 5-year graft survival.

DC could play an important role in determining the balance between transplant tolerance and immunity.[48] It has been suggested that immunological monitoring of peripheral blood DC subset numbers and their ratio might identify transplant recipients in whom the immunosuppressive load can be safely tapered.[23] It is assumed that blood DC in healthy individuals display an immature phenotype and induce T cell unresponsiveness.[49, 50] Immature DC are specialised in the capture of antigens, and transport them from peripheral tissues to secondary lymph nodes. Both donor and recipient DC could play a role in allograft rejection. Donor DC are transferred with the graft and can directly interact with recipient T cells. Recipient DC in peripheral lymphoid organs can take up soluble donor antigens, infiltrate the graft, and present the antigens to T cells.[51] Little is known about circulating DC after withdrawal of immunosuppression in kidney transplant recipients. Several studies reported interference of immunosuppressive drugs with DC numbers, differentiation and maturation of DC.[23, 29-31, 33] Our study showed that withdrawal of AZA or MMF had no effect on the absolute numbers of total DC, mDC and pDC and their ratio. Although, several problems related to monitoring DC phenotype and subsets in peripheral blood were reported, for example, variability in technique, absence of reference standards, incomplete data regarding the influence of disease, medication, and patient-related factors on blood DC subsets.[52] Additionally, some patients had mDC and pDC numbers that differed from the majority of patients. Nevertheless, interestingly, DC subsets shifted towards more mature DC phenotype after discontinuation of AZA or MMF, suggesting that AZA or MMF hinders the phenotypic maturation status of DC. In agreement with our data, it has been reported that MMF effects phenotypic DC maturation in both mice and *in vitro* models.[53, 54] In MMF-treated DC, a lower expression of CD40, CD80, CD86, CD83, and CD54 was observed, suggesting an inhibitory effect of MMF on DC maturation.[53] Also a dose-dependent inhibition of mixed lymphocyte reaction with AZA-treated DC was reported.[55] MMF is an inhibitor of the enzyme inosine monophosphate dehydrogenase (IMPDH), which is involved in *the novo* synthesis of guanosine nucleotides. The inhibitory effect of MMF on DC maturation could be caused by an imbalance between cyclic guanosine monophosphate and cyclic adenosine monophosphate

(cAMP) in DC.[56] Because both mDC and pDC circulate in peripheral blood[57], those data support our results that after discontinuation of AZA and MMF DC shifted towards a more mature phenotype.

In conclusion, recipients of an HLA-identical LR renal transplant can be safely withdrawn from AZA or MMF. The number of donor, 3rd-party, and TET-reactive IL-10 pc and maturation of DC was suppressed by AZA and MMF, suggesting that these immunosuppressive drugs may hinder downregulation of the general immune reactivity, including allogeneic responses.

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Chapter 11

A multiplex bead array analysis to monitor donor-specific cytokine responses after withdrawal of immunosuppression in HLA-identical living-related kidney transplant recipients

Multiplex bead array after HLA-identical living-related kidney transplantation

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ABSTRACT

Introduction: Immune reactivity after HLA-identical living-related (LR) kidney transplantation can be caused by minor histocompatibility antigen and non-HLA antigen mismatches between donor and recipient. In our center, HLA-identical LR kidney transplant recipients receive azathioprine (AZA) or mycophenolate mofetil (MMF) in combination with corticosteroids for one year after transplantation. Thereafter, AZA or MMF was withdrawn and the patients were treated with steroid monotherapy as maintenance therapy. We questioned whether withdrawal of AZA or MMF affected the donor-specific lymphocyte proliferation and cytokine production.

Methods: Donor and 3rd-party T-cell reactivity were determined by mixed lymphocyte reactions and by cytokine production using multiplex bead array technique.

Results: The donor and 3rd-party proliferative capacity were not affected after withdrawal of AZA or MMF. Thirteen of 17 cytokines were detected by the multiplex bead array technique. No differences were observed after 3rd-party induced cytokine production after withdrawal of AZA or MMF. However, production of donor-specific IFN-γ and MIP-1β increased after discontinuation of AZA or MMF, but no clinically relevant acute rejection was observed.

Conclusions: After HLA-identical LR kidney transplantation, donor-specific cytokine responses can be found when AZA or MMF therapy is discontinued. The clinical relevance of this phenomenon is still not evident.

INTRODUCTION

After solid organ transplantation, patients receive life-long immunosuppressive medication to suppress immune reactivity directed to the transplanted organ.[1] Considering the severe side effects of immunosuppressive medication, minimization of immunosuppression is warranted in transplant recipients without inducing rejection of the donor graft. It has been reported that only a minority of HLA-mismatched kidney transplant recipients could safely discontinue their immunosuppressive medication completely.[2-4] Recently, we described the possibility that HLA-identical living-related (LR) kidney transplant recipients can be withdrawn from their AZA and MMF dose.[5]

Theoretically, after HLA-identical LR kidney transplantation, differences in minor histocompatibility antigens (mHAgs) between donor and recipient may induce an immune response that might lead to graft rejection. mHAgs are intracellular peptides derived from non-HLA-encoded gene products that are presented on the cell surface of HLA class I and class II molecules, and can be recognised by T cells.[6] Those antigens are inherited according to the Mendelian segregation theory.[7] Indeed, after HLA-identical sibling bone marrow transplantation, immune reactivity is caused by mismatches in mHAgs between recipient and donor that may result in graft-*versus*-host disease.[8, 9] Therefore, discontinuation of immunosuppression in HLA-identical LR kidney transplant recipients may disturb the immunological balance and lead to rejection. Accurate monitoring of immune parameters is thus warranted (e.g. by measuring cytokine activity). Pro-inflammatory cytokines have been associated with increased immune reactivity and allograft rejection, while anti-inflammatory cytokines have been associated with downregulation of immune reactivity.[10]

Cytokines can be measured by several techniques, such as real-time polymerase chain reaction (PCR) for mRNA cytokine levels, and flow cytometry for intracellular cytokines, Elispot assays for frequency of cytokine producing cells, and enzyme-linked immunosorbent assays (ELISA) for cytokine concentrations.[11, 12] A recent developed assay to measure cytokine levels is the multiplex bead array technique. The advantage of this assay is that it provides simultaneous detection of multiple cytokines in serum, plasma or cell culture supernatants. The multiplex bead array technique captures cytokines by spectrally distinct beads, and uses fluorescent-labeled antibodies for detection of cytokines; samples are analysed on a compact flow cytometer.[13]

So far, there is no data available after clinical organ transplantation on simultaneous detection of donor-specific cytokine production in cell culture supernatants using a multiplex bead array technique. In our transplant center, HLA-identical LR kidney transplant recipients receive AZA or MMF in combination with steroids for 1 year after transplantation. From 1 year after transplantation, AZA or MMF are discontinued.[5] In the present study, we wondered whether withdrawal of AZA or MMF in HLA-identical LR kidney transplant recipients affected the donor-specific and 3rd-party specific cytokine production and proliferation.

MATERIALS AND METHODS

HLA-identical living-related kidney transplant recipients

The ethical review committee of our center approved the protocol that was conducted according to local requirements. After informed consent, we studied seven HLA-identical LR kidney transplant recipients, who were more than 2 years after transplantation with stable serum creatinine levels and no proteinuria (<0.5 g/L). At inclusion, patients received 50 mg/day AZA (n=6) or 500 mg/day MMF (n=1) in combination with 5 mg/day prednisolone (dual therapy). Thereafter, AZA and MMF dose were gradually tapered over a period of 4 months and the patients were kept on steroid monotherapy for at least one year. The characteristics of the patients are described in Table 1.

Table 1: Demographics of HLA-identical living-related kidney transplant recipients

	Patients (n=7)
Age (years) (at dual therapy)	53 (28-63)[1]
Gender (male/female)	5/2
Primary kidney disease	Patient 1: Medullary cystic disease
	Patient 2: Acute tubular necrosis
	Patient 3: IgA nephropathy
	Patient 4: Hypertension
	Patient 5: Von Hippel Lindau
	Patient 6: Meningococcal sepsis
	Patient 7: Reflux nephropathy
Time after transplantation (years)	6.3 (2.5-8.3)
# kidney transplantation: 1st / 2nd	6 / 1
PRA [2] (%)	2 (0-71)
Immunosuppression	AZA + prednisolone (n=6)
	MMF + prednisolone (n=1)

[1]Data are presented as: median (range); [2]PRA, panel reactive antibodies

Peripheral blood mononuclear cells sampling

At dual therapy and monotherapy, we received 35 ml peripheral blood to perform mixed lymphocyte reactions (MLR) and cytokine analysis. Peripheral blood mononuclear cells (PBMC) from recipient and donor were isolated from heparinized blood by density gradient centrifugation using Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden). PBMC were collected from the interphase, washed twice with RPMI-1640-DM (Cambrex, Verviers, Belgium) supplemented with 100 IU/ml of penicillin (Cambrex) and 100 µg/ml of streptomycin (Cambrex). Thereafter, PBMC were stored in RPMI-1640-DM containing 15% foetal calf serum (FCS) and 10% dimethyl sulphoxide (MERCK, Germany) at –140°C until use.

MLR and collection of supernatant

One hundred microliters of $5x10^4$ patient PBMC in complete culture medium [(RPMI-1640-DM (Cambrex, Verviers, Belgium) supplemented with filtered [0.20-µm sterile syringe filter, Corning Incorporated, Corning, NY) 10% heat inactivated pooled human serum, 100 IU/ml of penicillin (Cambrex) and 100 µg/ml of streptomycin (Cambrex)] were stimulated with 100 µl irradiated (45 Gy) $5x10^4$ PBMC derived from the donor or completely HLA-mismatched

3rd-party PBMC in a 96-well round bottom plate (Nunc, Roskilde, Denmark) for 7 days. To control the influence of irradiation, patient PBMC were incubated with its own irradiated patient PBMC (autologous response). Proliferation was measured by incorporation of ^{3}H-thymidine added during the last 8 h of culture. At day 6, supernatants were harvested and stored at –80°C until use.

Cytokine analysis

MLR supernatants were simultaneously tested for 17 cytokines (interleukin [IL]-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p70), IL-13, IL-17, interferon [IFN]-γ, tumor necrosis factor [TNF]-α, granulocyte colony-stimulating factor [G-CSF], granulocyte-macrophage colony-stimulating factor [GM-CSF], monocyte chemotactic protein [MCP]-1, and macrophage inflammatory protein [MIP]-1β) with the Bio-Plex Human Cytokine 17-Plex Panel following the manufacturer's description (Bio-Rad Laboratorius, Veenendaal, The Netherlands). MLR supernatants were analyzed using a Bio-Plex array reader equipped with Bio-Plex software (Bio-Rad Laboratories). Detection limit was determined according to a standard curve (cytokine [lower detection limit-upper detection limit]): IL-1β [3-10000 pg/ml]; IL-2 [3-20000 pg/ml]; IL-4 [5-1000 pg/ml]; IL-5 [5-10000 pg/ml]; IL-6 [10-10000 pg/ml]; IL-7 [5-10000 pg/ml]; IL-8 [2-30000 pg/ml]; IL-10 [10-8000 pg/ml]; IL-12(p70) [10-20000 pg/ml]; IL-13 [5-400 pg/ml]; IL-17 [5-7000 pg/ml]; IFN-γ [30-20000 pg/ml]; TNF-α [20-20000 pg/ml]; G-CSF [10-8000 pg/ml]; GM-CSF [20-10000 pg/ml]; MCP-1 [10-7000 pg/ml], MIP-1β [10-9000 pg/ml]. First, we tested which day is representative for optimal cytokine production of most cytokines in an HLA mismatched setting. In MLR supernatant, by day 6 most cytokines were detectable. Thereafter, we analysed the MLR supernatants at day 6 from the HLA identical LR renal transplant recipients. Cytokine concentrations ranging between the lower and higher detection limit were only analysed. The autologous response was subtracted from donor and from 3rd-party response to control the influence of irradiation.

mHAgs typing after HLA-identical LR kidney transplantation

DNA from donor and recipient were isolated using the QIAamp® DNA Mini Kit.[14] Patient and donor PBMC were typed for 11 known mHAgs: HA-1, HA-2, HA-3, HA-8, HB-1, ACC-1, ACC-2, HwA-9, HwA-10, UGT2B17, and HY.

Statistical analysis

The Wilcoxon signed rank test (two-sided P-values) was used to compare differences between donor-reactive responses and 3rd-party reactive responses and to compare differences between dual therapy and monotherapy. For statistical analysis, GraphPad statistical program was used (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Clinical results

One year after withdrawal of AZA or MMF, HLA-identical LR kidney transplant recipients had stable serum creatinine levels (dual therapy: median, 108 μmol/L; range, 62-135 *versus*

monotherapy: median, 114 μmol/L; range, 63-141) and no proteinuria (<0.5 g/L). None of the patients experienced acute rejection episodes or infections with cytomegalovirus or Epstein-Barr virus.

MLR

No difference was observed in donor-reactive MLR before (median, 231 cpm [range, 86-5527]) and after withdrawal of AZA or MMF (monotherapy: median, 164 cpm [77-6050]; p=0.99). The donor-reactive MLR was comparable to the autologous MLR during dual therapy (donor-reactive MLR: median, 231 cpm [86-5527]; autologous MLR: 113 cpm [58-3377]; p=0.30) and during monotherapy (donor-reactive MLR: median, 164 cpm [77-6050]; autologous MLR: median, 165 cpm [76-3410]; p=0.30). Before (p=0.02) and after (p=0.02) withdrawal of AZA or MMF, donor-reactive MLR was significantly lower than the 3rd-party reactive MLR. Discontinuation of AZA or MMF had no influence on the 3rd-party reactive MLR (dual therapy: median, 7760 cpm [603-42810]; monotherapy: median, 8067 cpm [201-29869]; p=0.99).

Cytokine analysis

MLR supernatants were analysed for the presence of 17 cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p70), IL-13, IL-17, IFN-γ, TNF-α, G-CSF, GM-CSF, MCP-1 and MIP-1β). Donor and 3rd-party reactive IL-4, IL-7, IL-10 and IL-12(p70) were below detection limit before and after withdrawal of AZA or MMF, while donor and third-party IL-5 and IL-13 levels were inconsistently detectable. Donor and 3rd-party reactive IL-8 production was too high to determine exact cytokine levels.

Table 2: Influence of AZA and MMF withdrawal on donor and 3rd-party specific responses[1] in PBMC from 7 HLA-identical LR kidney transplant recipients

Cytokine	Dual therapy		Monotherapy steroids	
	Donor-specific response (pg/ml)	3rd-party specific response (pg/ml)	Donor-specific response (pg/ml)	3rd-party specific response (pg/ml)
IL-1β	3 (0-432)[3]	12(0-541)	1 (0-839)	0 (0-269)
IL-2	0 (0-23)	34 (0-78)	5 (0-21)	26 (0-51)
IL-6	30 (0-2092)	60 (0-2297)	0 (0-8475)	3 (0-1854)
IL-17	0 (0-31)	12 (0-130)	9 (0-35)	13 (0-31)
IFN-γ	0 (0-171)	121 (0-550)	11 (0-301)	100 (0-194)
TNF-α	0 (0-525)	165 (28-216)	17 (0-416)	93 (0-1085)
G-CSF	0 (0-719)	0 (0-1067)	0 (0-545)	0 (0-77)
GM-CSF	0 (0-102)	54 (0-119)	31 (0-105)	32 (0-90)
MCP-1	0 (0-357)	210 (0-824)	325 (0-1056)	121 (0-556)
MIP-1β	0 (0-269)	1033 (171-3317)	266 (0-3184)	961 (94-7990)

Data are presented as median (range). AZA, azathioprine; MMF, mycophenolate mofetil; PBMC, peripheral blood mononuclear cells; LR, living-related; IL, interleukin; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; MCP-1, monocyte chemotactic protein-1; MIP-1β, macrophage inflammatory protein-1β. 1IL-4, IL-7, IL-10, and IL-12(p70) production were nondetectable. IL-8 production was too high to determine exact cytokine levels. Donor and third-party IL-5 and IL-13 levels were inconsistently detectable.

The autologous response was subtracted from donor and from third-party responses. We detected 10 donor- and 3rd-party specific cytokine responses before and/or after discontinuation of AZA or MMF in all patients. These cytokines were IL-1β, IL-2, IL-6, IL-17, IFN-γ, TNF-α, G-CSF, GM-CSF, MCP-1 and MIP-1β (Table 2).

Withdrawal of AZA or MMF had no influence on the production of donor and 3rd-party specific IL-1β, IL-2, IL-6, IL-17, TNF-α, G-CSF, GM-CSF and MCP-1 (Table 2). The production of donor-specific IFN-γ was higher during monotherapy (median, 11 pg/ml; range, 0-301) than during dual therapy (median, 0 pg/ml; range, 0-171; p=0.06; Figure 1A). After withdrawal of AZA or MMF, we observed that in five of seven patients, the production of donor-specific IFN-γ had increased. Donor-specific IFN-γ responses were lower than the 3rd-party specific IFN-γ responses during dual therapy (p=0.06). No difference was observed for the 3rd-party specific IFN-γ response (Table 2; Figure 1B).

In addition, the production of donor-specific MIP-1β was also higher after withdrawal of AZA or MMF (dual therapy: median, 0 pg/ml [0-269]; monotherapy: median, 266 pg/ml [0-3184]; p=0.06; Figure 1C). In five of the seven patients, the donor-specific MIP-1β

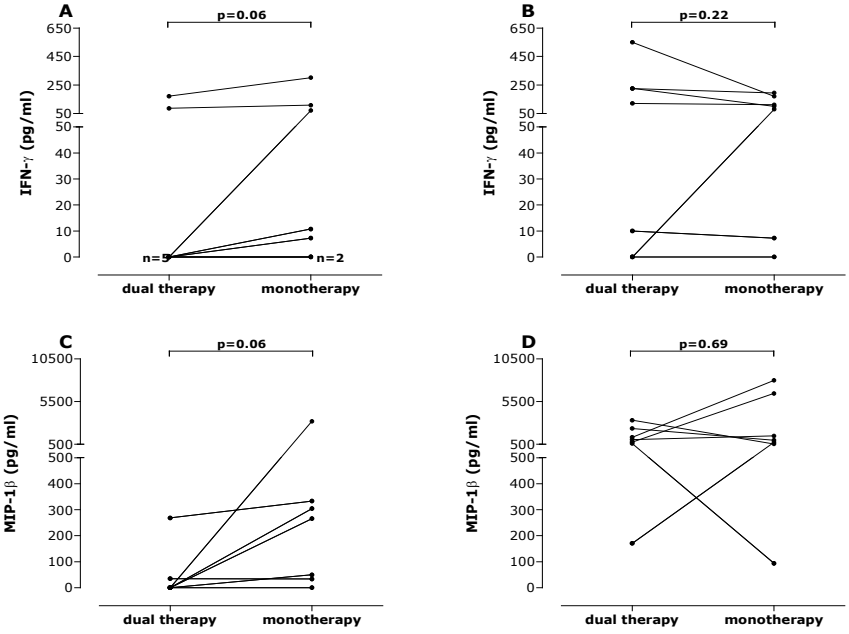

Figure 1: Donor (A, C) and 3rd-party (B, D) specific cytokine production of IFN-γ and MIP-1β in MLR cultures from PBMC of HLA-identical LR kidney transplant recipients (n=7) during dual therapy (AZA or MMF + steroids) and during steroid monotherapy.

production had increased after withdrawal of immunosuppression. Donor-specific MIP-1β responses were lower than the 3rd-party specific IFN-γ responses both during dual therapy (p=0.02) and during monotherapy (p=0.02). The production of 3rd-party specific MIP-1β was not affected (Table 2; Figure 1D).

Table 3: Known mismatched mHAgs and donor-specific IFN-γ and MIP-1β production after HLA-identical LR kidney transplantation

Pat	Gender		Mismatched mHAgs in the HLA-identical donor	HLA-restriction molecule present	Donor-specific IFN-γ production (pg/ml)		Donor-specific MIP-1β production (pg/ml)	
	Pat	Don			dual therapy	monotherapy	dual therapy	monotherapy
1	F	F	HA-1H, HA-3M, HB-1Y		171	301	0	3184
2	M	F	HB-1Y		0	0	0	304
3	M	M	No difference		87	108	0	266
4	M	F	HA-2V [1], HA-8R, HB-1H	A2	0	71	35	33
5	M	F	HA-3M, HA-8R, HwA-9R	A3	0	11	269	333
6	F	F	No difference		0	7	0	50
7	M	F	HB-1Y		0	0	0	0

mHAgs, minor histocompatibility antigens; IFN-γ, interferon-γ; MIP-1β, macrophage inflammatory protein-1β; LR, living-related. [1]Mismatched mHAgs with the correct HLA-restriction molecule are presented as underlined

Relation between production of donor-specific cytokines and mHAg mismatches

We analysed whether the presence (≥1 pg/ml) of donor-specific IFN-γ and MIP-1β production was related to mHAg mismatches determined between donor and recipient.

From the seven patient-donor combinations, five couples (patient 1, 2, 4, 5, 7) demonstrated at least one mHAg mismatch (Table 3). Two out of these five couples (patient 4 and 5) had an mHAg mismatch in combination with the correct HLA-restriction molecule (i.e. the HLA molecule in which the donor mHAgs could be presented to T cells). Remarkably, in patient 4 and 5, positive donor-specific MIP-1β responses were found both before and after withdrawal of AZA and MMF, while during monotherapy donor-specific MIP-1β production was detectable in six patients independent of mHAg mismatches. We could not find differences between IFN-γ and mHAg mismatches. Additionally, no relation was found between production of donor-specific IL-1β, IL-2, IL-6, IL-17, TNF-α, G-CSF, GM-CSF, and MCP-1 and the presence of known mHAg mismatches (data not shown).

DISCUSSION

After kidney transplantation, life-long use of immunosuppression is deemed necessary to avoid rejection of the transplanted kidney.[1, 15] However, the necessity for long-term use of immunosuppression in HLA-identical LR kidney transplant recipients is yet unknown. Considering the severe side effects of immunosuppression, tapering or even withdrawal of immunosuppression in these transplant recipients might be beneficial. Theoretically, withdrawal of immunosuppression in solid organ transplant recipients might result into increased donor-reactive T-cell responses.[16-19] In the present study, none of the patients had an acute rejection episode and renal function remained stable when AZA or MMF was discontinued. We determined the

donor-reactive MLR response after HLA-identical LR kidney transplantation; the donor-reactive MLR response was not higher than the autologous response. Apparently, priming of mHAgs as detected by MLR did not occur.

From the 17 cytokines analysed, 13 cytokines could be detected from which IL-8 was too high to determine exact cytokine levels, while donor and third-party IL-5 and IL-13 levels were inconsistently detectable. We observed that withdrawal of AZA or MMF had no significant influence on eight out of the remaining 10 detectable donor and 3rd-party specific cytokines. Interestingly, the production of donor-specific IFN-γ and MIP-1β was higher after withdrawal of AZA or MMF, while the production of 3rd-party specific responses remained stable. This suggests that AZA or MMF suppresses donor-specific production of IFN-γ and MIP-1β. AZA and MMF are anti-proliferative agents that may have an effect on cytokine production.[20, 21] We are aware of our small cohort of patients. Therefore, future studies are needed to confirm our results.

Pro-inflammatory cytokines (IL-2, IFN-γ, TNF-α, Regulated upon Activation, Normal T-cell Expressed, and Secreted [RANTES], MIP-1α and MIP-1β) have been associated with increased immune reactivity and allograft rejection, while anti-inflammatory cytokines (IL-4, IL-5, IL-10 and IL-13) have been postulated to downregulate immune responses.[22] In the present study, we found higher donor-specific IFN-γ and MIP-1β production after withdrawal of AZA or MMF. Both cytokines can be produced by T-helper (Th) 1 cells and natural killer (NK) cells.[23-25] Apparently, the increase in IFN-γ and MIP-1β production capacity did not result in rejection.

It has been suggested that the presence of panel of reactive antibodies (PRA)-reactivity was associated with long-term graft loss in kidney transplants from HLA-identical sibling donors, which could reflect immune reactivity directed to non-HLA antigens or mHAgs.[26] In the present study, we found no correlation between PRA before HLA-identical LR kidney transplantation and increased donor-specific IFN-γ and MIP-1β production during withdrawal of AZA and MMF. Additionally, the presence of PRA before transplantation did not result in acute rejection after discontinuation of AZA and MMF.

Donor-specific MIP-1β during dual therapy was related with mHAg mismatches in combination with the correct HLA-restriction molecule, suggesting that those responses were caused by those specific mHAg mismatches. This observation needs to be confirmed in a larger cohort of HLA-identical LR kidney transplant recipients. Apparently, AZA or MMF does not suppress the donor-specific MIP-1β production caused by mHAg mismatches presented in the correct HLA-restriction molecule, but again, this did not result in rejection.

In conclusion, donor reactivity can be detected in HLA-identical LR kidney transplant recipients using multiplex bead array technique. AZA and MMF suppress donor-specific IFN-γ and MIP-1β production in HLA-identical LR kidney transplant recipients. However, the clinical relevance of this finding remains unclear.

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Chapter 12

Summary and Conclusions

After HLA-identical living-related (LR) kidney transplantation, less acute rejection and better graft survival occur compared to recipients of an HLA-mismatched kidney transplant.[1] This is explained that all major HLA molecules are identical between donor and recipient and only mismatches in minor histocompatibility antigens (mHAgs) and other non-HLA antigens may exist. The clinical relevance of mHAgs on transplant outcome has been demonstrated after bone marrow transplantation.[2, 3] However, their relevance is yet not known after solid organ transplantation. Theoretically, both mismatches in mHAgs and other non-HLA antigens between donor and recipient may induce graft rejection. Consequently, HLA-identical LR renal transplant recipients still receive immunosuppression.

In **Chapter 1**, we provide a general introduction on immune monitoring after kidney transplantation. Non-invasive cellular assays using patients' PBMC to determine immune reactivity directed to donor antigens could be helpful to identify transplant recipients in whom the immunosuppressive load can be safely reduced. Donor-reactive responses were determined by mixed lymphocyte reaction (MLR), CTL precursor frequency (CTLpf), helper T-lymphocyte precursor frequency (HTLpf) and Elispot assays before and during tapering of immunosuppression. In addition, a short overview concerning the development and side-effects of immunosuppressive drugs are given.

The **Aim of this thesis** was to clarify the clinical relevance of donor-reactive T-cell responses directed to mHAgs and non-HLA antigens after HLA-identical LR kidney transplantation and during tapering of immunosuppression in this patientgroup **(Chapter 2)**.

In **Part I**, we studied donor-reactive T-cell responses during the first year after HLA-identical LR kidney transplantation. After HLA-mismatched organ transplantation, studies showed that the donor-reactive T-cell responses, determined by either MLR, CTLpf, HTLpf or IFN-γ Elispot assays, decrease during the first year after transplantation.[4-8] In **Chapter 3**, we demonstrated that, during immunological quiescence, donor-reactive IFN-γ and GrB producing cells can be found in peripheral blood mononuclear cells (PBMC) from recipients of an HLA-identical LR kidney transplant both before and after HLA-identical LR kidney transplantation. We found no decrease in the number of donor and 3rd-party reactive IFN-γ and GrB producing cells. These results were comparable with our previous data in a smaller cohort of HLA-identical LR kidney transplant patients.[9] In addition, the number of donor-reactive IFN-γ and GrB producing cells were not related with known mHAg mismatches (HA-1, HA-2, HA-3, HA-8, HB-1, ACC-1, ACC-2, HwA-9, HwA-10, UGT2B17 and HY) between donor and recipient.

Subsequently, we questioned whether the number of cytokine producing cells (IFN-γ, GrB, IL-13 and IL-10) directed to alloantigens in PBMC was different between HLA-identical LR kidney transplant recipients and HLA-mismatched LR kidney transplant recipients, who were approximately one year after transplantation. Healthy individuals served as controls. **Chapter 4** showed that alloreactive cytokine responses (IFN-γ, GrB and IL-13) from healthy individuals were higher compared to HLA-mismatched and HLA-identical LR kidney transplant recipients. Additionally, only the number of donor-reactive GrB producing cells was higher in the HLA-mismatched group compared to the HLA-identical group. Interestingly, in the HLA-identical group, the number of donor-reactive IL-10 producing cells was significantly higher than their autologous control. These frequencies were also higher than the frequencies in the HLA-mismatched group and in healthy individuals. Additionally, donor-reactive cytokine responses could not be related to known mHAg mismatches between donor and recipient.

IL-10 is an anti-inflammatory cytokine that have been associated with downregulation of immune responses and may play a role in preventing graft rejection.[10-12] After HLA-identical LR kidney transplantation, several studies showed the presence of donor-reactive IL-10 that could reflect allograft tolerance.[13, 14] Several cells produce IL-10 after stimulation with donor antigens, such as monocytes, B cells, activated Th2 cells and T regulatory 1 (Tr1) cells.[10] Monocytes do not interfere in our Elispot assays, because only non-adherent cells were used. Also B cells played no role in our assays, because no difference in donor-reactive IL-10 response was found between PBMC depleted for B cells and PBMC not depleted for B cells (data not shown). Beside IL-10, activated Th2 cells can also secrete IL-4, IL-5 and IL-13, and Tr1 cells produce transforming growth factor (TGF)-β.[15, 16] Because high numbers of donor-reactive IL-10 producing cells were found in combination with low numbers IFN-γ, IL-13 and GrB producing cells, we assume that not Th2 cells, but Tr1 cells could play a role after HLA-identical LR kidney transplantation. We suggests that IL-10 might play a beneficial role in active downregulation of immune reactivity against donor antigens during immunological quiescence.

In **Chapter 5**, the expression profile of 42 cytokines was screened by protein-array analysis in supernatant from donor-stimulated mixed lymphocyte cultures from PBMC of HLA-identical LR kidney transplant recipients. We observed that in 38 out of 42 (90%) proteins, donor-reactive response was higher than autologous control. We assumed that those responses could be directed to mismatched mHAgs or other non-HLA antigens.

In addition to immunological monitoring of donor-reactive T-cell responses in transplant recipients, it has been suggested that monitoring of circulating DC subsets [myeloid DC (mDC) and plasmacytoid DC (pDC)] and their ratio in peripheral blood of transplant recipients might be an useful tool for identifying transplant recipients in whom the immunosuppressive load can be safely tapered.[17] Because, it is practical and probably more accurate to measure sequential blood samples of one patient in one flow cytometric session, we studied the influence of cryopreservation on pDC/mDC ratio in healthy individuals and kidney transplant recipients **(Chapter 6)**. We observed in both study groups that manipulation of PBMC by Ficoll isolation and cryopreservation affected the pDC/mDC ratio compared to fresh blood samples. Several studies demonstrated that immunosuppression may influence DC numbers.[18-20] Therefore, we compared our results with PBMC from heart failure patients who did not receive immunosuppressive medication. The pDC/mDC ratio was also higher after cryopreservation of PBMC from heart failure patients compared to fresh blood cells, suggesting that the affected ratio was caused by blood manipulations, and not by immunosuppression. In addition, Ficoll isolation and cryopreservation of PBMC affected the maturation status of DC towards a more mature phenotype. These data imply that fresh blood samples should be used for monitoring purposes.

Considering the severe side effects of immunosuppression, minimizing of immunosuppression in transplant recipients might be beneficial. Theoretically, discontinuation of immunosuppression in organ transplant recipients might result into an increased donor-reactive T-cell response.[21-24] Complete discontinuation of immunosuppression was reported in a few renal transplant recipients long after transplantation with stable graft function and without clinical signs of rejection.[25-27] However, after HLA-identical LR kidney transplantation there is no literature available about the necessity of immunosuppression in these patients and whether tapering of immunosuppression affected donor-reactive T-cell responses **(Part**

II). Therefore, we tapered the azathioprine (AZA) dose to 50% of the original dose in HLA-identical LR kidney transplant recipients, more than 2-years after transplantation, and studied the effect on renal function and T-cell reactivity **(Chapter 7 and 8)**. After tapering the AZA dose, HLA-identical LR kidney transplant recipients remained free from acute rejection episodes and renal function was unaffected. We found that before tapering of AZA, the number of donor-reactive GrB producing cells was comparable to the number of GrB producing cells after tapering the AZA dose. Interestingly, before reduction of AZA dose, only 20% of the patients' responded to donor antigens, whereas 57% of the patients' PBMC responded to donor antigens after tapering of AZA. After 3rd-party stimulation, the number of GrB producing cells was increased after tapering the AZA dose **(Chapter 7)**. Tapering of AZA did not increase the number of donor, 3rd-party and tetanus toxoid (TET)-reactive IFN-γ and IL-13 producing cells **(Chapter 8).** In addition, donor-reactive cytokine responses measured before and after tapering of AZA could not be related to the number of known mHAg mismatches between donor and recipient.

Because none of those patients experienced an acute rejection episode and renal function remained unaffected and no increase in the donor-reactive T-cell response was observed after tapering of the AZA dose, we assumed that the immunosuppressive dose could be reduced further **(Chapter 9 and 10)**. We observed that withdrawal of AZA or MMF was successful in 85% (23/27) of the patients **(Chapter 9)**. None of those patients had an acute rejection episode and renal function remained unaffected during a 24-months follow-up period. Also other clinical parameters, such as systolic, diastolic blood pressure, the number of antihypertensive drugs taken, serum total, LDL or HDL cholesterol levels, haemoglobulin, thrombocytes or leukocytes, showed no significant changes between inclusion and 24-months on monotherapy prednisolone. 15% (4/27) of the patients developed recurrence of their original disease. This percentage was comparable to the recurrence of disease described elsewhere in HLA-identical LR kidney transplant recipients who used full dose immunosuppression.[28]

We observed no significant differences in the number of donor, 3rd-party and TET-reactive IFN-γ and GrB producing cells after withdrawal of AZA or MMF therapy **(Chapter 10)**. Remarkably, higher numbers of donor, 3rd-party and TET-reactive IL-10 producing cells were found during monotherapy prednisolone. Apparently, IL-10 was suppressed by AZA and MMF. AZA and MMF are anti-proliferative agents, and may also have an effect on cytokine production of lymphocytes.[29, 30] Furthermore, discontinuation of AZA or MMF had no influence on the absolute numbers of total DC, mDC and pDC, and their ratio. The maturation status of DC subsets was shifted towards a more mature phenotype, suggesting that AZA and MMF affected phenotypic maturation of DC. In agreement with our results, studies have reported that AZA and MMF may affect phenotypic maturation of DC.[31-33]

Thereafter, we wondered whether we could detect differences in donor reactivity after discontinuation of AZA and MMF in lymphocyte proliferation and cytokine production using multiplex bead array technique **(Chapter 11)**. We found that donor and 3rd-party proliferative capacity were not affected after withdrawal of AZA or MMF. Thirteen out of 17 donor and 3rd-party reactive cytokines were detected during dual therapy and during monotherapy prednisolone. Four cytokines were not detectable. Only the production of donor-reactive IFN-γ and MIP-1β was higher after withdrawal of AZA or MMF, while the production of 3rd-party reactive responses remained stable. We concluded that donor reactivity can be detected in HLA-identical LR kidney transplant recipients using multiplex bead array technique. AZA and MMF suppressed the production of donor-reactive IFN-γ and MIP-1β in HLA-identical LR kidney transplant recipients, and not the 3rd-party reactive response. The

clinical relevance of this finding is yet unclear.

Considering the successful clinical and laboratory results after tapering and even discontinuation of AZA or MMF in HLA-identical LR kidney transplant recipients, we propose to withdrawal the immunosuppressive medication (calcineurin inhibitors [CNI], AZA or MMF) to prednisolone 5 mg/day in these patients who are at least one year after transplantation. Low dose steroid monotherapy should be sufficient provided that they have stable renal function and no proteinuria. Close surveillance of patients for recurrence of their original disease is recommended for potential early therapeutic intervention. Whether complete withdrawal of corticosteroids may be possible in HLA-identical LR kidney transplant recipients remains to be investigated. Interestingly, in our study group, two HLA-identical LR kidney transplant recipients are also withdrawn from corticosteroid for more than 3 years. They did not develop acute rejection and have stable graft function (data not shown).

In summary, this thesis demonstrated that donor-reactive T-cell responses can be found after HLA-identical LR kidney transplantation, but those responses were not related to known mHAg mismatches (HA-1, HA-2, HA-3, HA-8, HB-1, ACC-1, ACC-2, HwA-9, HwA-10, UGT2B17 and HY) **(Chapter 3, 4, 8, 10, 11)**. In line with our study, Heinold *et al.*[34] reported that mHAg mismatches between donor and recipient after cadaveric and living-related kidney transplantation had no significant influence on the 5-years death-censored allograft survival. Other studies reported an association with mHAgs and acute rejection, chronic allograft nephropathy and graft failure. However, they analysed these associations in patients who received a deceased kidney and not in HLA-identical siblings.[35-38] So, one cannot exclude the possibility that the association was caused by HLA mismatches, as HLA-Cw, HLA-DQ and DP, rather than mHAg mismatches in those patients.[39-42]

Proinflammatory cytokines, such as IFN-γ, IL-2 and TNF-α, have been postulated to promote allograft rejection, while anti-inflammatory cytokines, IL-4, IL-5, IL-10 and IL-13, have been associated with downregulation of the immune response.[43-45] In our studies, donor-reactive T-cell responses could be detected in PBMC from HLA-identical LR kidney transplant recipients using Elispot assays during immunological quiescence **(Chapter 3, 4, 7, 8, 10)**. Interestingly, we found a negative correlation (r_s=-0.41; p=0.03) between the number of donor-reactive IL-10 producing cells and time after HLA-identical LR kidney transplantation before tapering of immunosuppression (Figure 1), while this was not observed for IFN-γ and GrB (data not shown). Withdrawal of AZA or MMF resulted in higher numbers of donor-reactive IL-10 producing cells in PBMC from HLA-identical LR kidney transplant recipients **(Chapter 10)**. This suggests the presence of immune reactivity against donor antigens during the first year after transplantation and during withdrawal of immunosuppression, which was actively downregulated by donor-reactive IL-10 in these patients. Several other studies showed also the presence of donor-reactive IL-10 in recipients of an HLA-identical LR kidney transplant that could reflect allograft tolerance.[13, 14, 46]

We felt reassured that none of the patients had an acute rejection episode during withdrawal of AZA and MMF. Therefore, the clinical relevance of the Elispot assay to predict acute rejection could not be demonstrated in this specific patient group.

In conclusion, in peripheral blood, donor-reactive T-cell responses can be found in PBMC from recipients of an HLA-identical LR kidney transplant. These responses are not related with the number of known mHAgs mismatches between donor and recipient. High numbers

of donor-reactive IL-10 producing cells were found. This may reflect active downregulation of immune reactivity against mHAgs and non-HLA antigens. HLA-identical LR kidney transplant recipients can be safely discontinued from their CNI, MMF and AZA dose after one year posttransplantation. Low dose corticosteroids is sufficient for stable graft function.

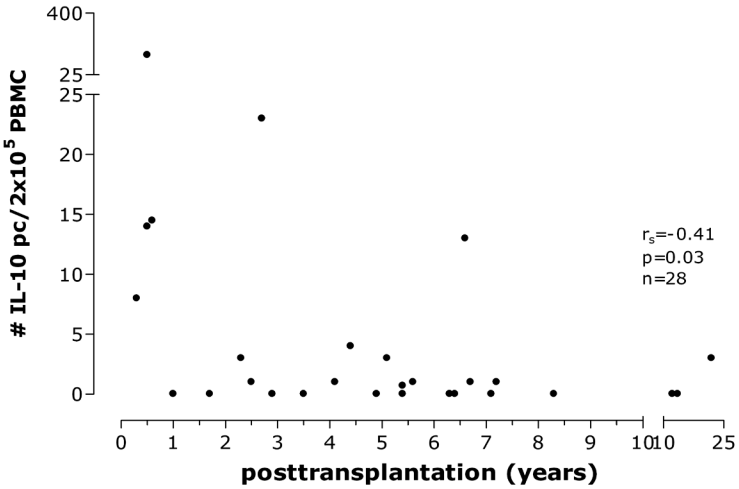

Figure 1: Relation between the number of donor-reactive IL-10 producing cells (pc) in PBMC from HLA-identical LR kidney transplant recipients and time after transplantation during high dose of immunosuppression.

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Hoofdstuk 13

Nederlandse samenvatting

Niertransplantatie is de beste behandeloptie voor patiënten met niet functionerende nieren. Hierbij wordt de zieke nier van de patiënt vervangen door een gezonde nier van de donor. Na transplantatie ontvangen patiënten medicijnen (immunosuppressiva) die het afweersysteem onderdrukken. Deze medicijnen worden gegeven om afstoting van de getransplanteerde nier te voorkomen. In het algemeen zal de patiënt zijn of haar hele leven deze medicijnen moeten innemen om acceptatie van de nier te handhaven. Langdurig gebruik van deze immuunsuppressieve geneesmiddelen kan echter leiden tot ernstige bijwerkingen, zoals infecties, nier- en leverfalen, suikerziekte, tumoren en hart- en vaatproblemen. Het is daarom wenselijk deze medicijnen in een minimale hoeveelheid voor te schrijven, zonder dat dit leidt tot afstoting van de getransplanteerde nier.

Transplantatie van een nier tussen individuen die genetisch niet identiek zijn, leidt tot afstoting wanneer de patiënt geen immunosuppressieve geneesmiddelen krijgt. Dit wordt veroorzaakt door een afweerreactie tegen lichaamsvreemde weefselantigenen op het celmembraan van de getransplanteerde nier. Deze weefselantigenen worden humane leukocyten antigenen (HLA; Human Leucocyte Antigens) genoemd. Wanneer alle HLA moleculen tussen ontvanger en donor identiek zijn, wordt de kans op afstoting aanzienlijk kleiner. Na HLA-identieke familieniertransplantaties zijn alle belangrijke HLA moleculen (HLA-A, B, Cw, DR, DP en DQ) tussen donor en ontvanger gelijk.

Ondanks dat bij HLA-identieke familieniertransplantaties alle HLA antigenen tussen donor en ontvanger gelijk zijn, kunnen er wel verschillen zijn in niet-HLA antigenen, zoals minor histocompatibiliteitsantigenen (mHAgs). mHAgs zijn genetisch overgeërfde peptiden (kleine stukjes eiwit) die afkomstig zijn uit polymorfe, intracellulaire eiwitten. Deze peptiden kunnen alleen worden gepresenteerd in het HLA molecuul, dat zich op het celoppervlak bevindt. mHAgs kunnen worden herkend door T cellen van de ontvanger en zij kunnen daarom een immuunrespons induceren. De klinische relevantie van mHAgs met betrekking tot afstotingsreacties is met name beschreven na beenmergtransplantatie.[1, 2] Hieruit bleek dat verschillen in mHAgs tussen ontvanger en donor tot een afweerreactie kan leiden, gericht tegen de ontvangercellen. De klinische relevantie van mHAgs na HLA-identieke familieniertransplantatie is echter nog onbekend. Gezien de resultaten bij beenmergtransplantaties, zou kunnen worden verondersteld dat verschillen in mHAgs of andere niet-HLA antigenen bij deze patiënten leiden tot afstoting van de getransplanteerde nier. Daardoor ontvangen deze patiënten nog steeds een onderhoudsdosering aan immunosuppressieve medicatie.

Vanwege de ernstige bijwerkingen van immunosuppressie, is het belangrijk om patiënten te identificeren bij wie de immunosuppressieve medicatie op een veilige manier kan worden verminderd. Dit kan door het monitoren van aanwezige immunologische reactiviteit gericht tegen donorcellen. **Hoofdstuk 1** beschrijft een algemene introductie over het monitoren van immunologische reactiviteit na niertransplantatie. Er worden niet-invasieve cellulaire testen beschreven die gebruik maken van patiënten- en donorcellen. Donorreactiviteit werd bepaald met 'gemengde lymfocyten kweek' (mixed lymphocyte reaction: MLR), frequentie van donorspecifieke helper (HTLpf) of cytotoxische T-lymfocyten (CTLpf), of Elispot testen. Deze technieken werden beschreven vóór de transplantatie en tijdens het verminderen van immunosuppressieve medicatie. Verder wordt in dit hoofdstuk een kort overzicht gegeven over de ontwikkeling van de medicatie en over bijwerkingen van immunosuppressiva.

Het hoofddoel van dit proefschrift was het onderzoeken van de klinische relevantie van donorgerichte T-cel reactiviteit tegen mHAgs na HLA-identieke familieniertransplantatie

en tijdens vermindering van immunosuppressie bij deze patiënten **(Hoofdstuk 2)**.

In het eerste deel hebben we de T-cel reactiviteit gericht tegen donorantigenen gedurende het eerste jaar na HLA-identieke familieniertransplantatie onderzocht. Studies na niet-HLA-identieke orgaantransplantatie hebben laten zien dat de T-cel reactiviteit gericht tegen donorcellen, gemeten met behulp van MLR, CTLpf en HTLpf, dalen gedurende het eerste jaar na transplantatie.[3-6] In **Hoofdstuk 3** wordt aangetoond dat donor-reactieve IFN-γ en GrB producerende cellen kunnen worden gemeten in perifere bloed mononucleaire cellen (PBMC) van ontvangers van een HLA-identieke familieniertransplantaat, zowel voor als na transplantatie. De donor- en de 3e-partij reactiviteit daalden niet gedurende het eerste jaar na transplantatie. Deze resultaten kwamen overeen met eerder gevonden resultaten bij een kleinere groep van HLA-identieke familieniertransplantatiepatiënten.[7] We vonden echter geen relatie met bekende mHAg verschillen (HA-1, HA-2, HA-3, HA-8, HB-1, ACC-1, ACC-2, HwA-9, HwA-10, UGT2B17 and HY) tussen donor en ontvanger en het aantal donor-reactieve IFN-γ en GrB producerende cellen.

Daarna vroegen we ons af of er een verschil bestaat in het aantal cytokinen producerende cellen (IFN-γ, GrB, IL-13 en IL-10) gericht tegen alloantigenen tussen HLA-identieke en niet-HLA-identieke familieniertransplantatiepatiënten. Gezonde individuen dienden hierbij als controle. **Hoofdstuk 4** laat zien dat alloreactieve cytokinen responsen (IFN-γ, GrB en IL-13) van gezonde individuen hoger waren vergeleken met beide patiëntengroepen. Ook vonden we dat het aantal GrB producerende cellen bij de niet-HLA-identieke groep hoger was dan de HLA-identieke groep. Opmerkelijk was dat het aantal donor-reactieve IL-10 producerende cellen bij de HLA-identieke groep significant hoger was dan de autologe reactiviteit. Deze frequentie was ook hoger dan de frequentie in de niet-HLA identieke groep en gezonde individuen. Er werd opnieuw geen relatie gevonden met het aantal cytokinen producerende cellen in PBMC van HLA-identieke patiënten en bekende mHAg verschillen tussen donor and ontvanger.

Vanuit de literatuur wordt beschreven dat IL-10 een anti-inflammatoir cytokine is dat immuunreactiviteit kan onderdrukken en mogelijk zelfs een rol speelt bij het voorkomen van afstoting van een donororgaan.[8-10] Studies na HLA-identieke familieniertransplantatie hebben laten zien dat de aanwezigheid van donorreactief IL-10 mogelijk tot tolerantie kan leiden.[11, 12] Verscheidene cellen produceren IL-10 na stimulatie met donorantigenen, zoals monocyten, B cellen, T helper (Th)-2 cellen en T regulatoire type 1 (Tr1) cellen.[8] Monocyten beïnvloedden de Elispot testen niet, omdat bij deze test alleen niet-adherente cellen waren gebruikt. Ook B cellen hadden geen invloed op de testen, omdat er geen verschil is gevonden in donor-reactieve IL-10 respons tussen PBMC zonder B cellen en PBMC met B cellen (data niet getoond). Naast IL-10 kunnen geactiveerde Th2 cellen ook IL-4, IL-5 en IL-13 produceren, terwijl Tr1 cellen transforming growth factor (TGF)-β produceren.[13, 14] Omdat hoge aantallen donor-reactieve IL-10 producerende cellen worden gevonden in combinatie met lage aantallen IFN-γ, GrB en IL-13 producerende cellen, wordt aangenomen dat niet Th2 cellen, maar Tr1 cellen een rol kunnen spelen na HLA-identieke familieniertransplantatie. Onze resultaten suggereren dat IL-10 een rol speelt bij het actief onderdrukken van immuunreactiviteit gericht tegen donorantigenen tijdens immunologische rust.

In **Hoofdstuk 5** hebben we met behulp van een eiwit-array in het supernatant van donorgestimuleerde MLR het expressieprofiel van 42 cytokinen onderzocht. We vonden bij 38 van de 42 (90%) eiwitten een hogere donor- dan de autologe reactiviteit. We veronderstellen dat deze responsen zijn gericht tegen mHAgs of andere niet-HLA antigenen.

In de literatuur wordt beschreven dat monitoren van circulerende dendritische cellen (DC) [myeloide DC (mDC) en plasmacytoide DC (pDC)] en de ratio pDC/mDC in het perifere bloed van transplantatiepatiënten mogelijk patiënten kan identificeren bij wie de immunosuppressieve geneesmiddelen kunnen worden verminderd.[15] Omdat het praktisch en mogelijk ook nauwkeuriger is om sequentiële bloedmonsters van één patiënt in één flowcytometrische meting te bepalen, hebben we onderzocht of de pDC/mDC ratio wordt beïnvloed door cryopreservatie van cellen van gezonde individuen en niertransplantatiepatiënten **(Hoofdstuk 6)**. In beide groepen vonden we dat de pDC/mDC ratio werd beïnvloed door het manipuleren van PBMC, zoals Ficoll isolatie en cryopreservatie. Er bestaan verscheidene studies die laten zien dat immunosuppressiva een effect kan hebben op de aantallen DC.[16-18] Daarom hebben we onze resultaten vergeleken met PBMC van patiënten met hartfalen die geen immunosuppressieve medicatie ontvangen. Ook bij deze patiënten was de pDC/mDC ratio hoger na cryopreservatie van PBMC vergeleken met de pDC/mDC ratio gemeten in verse bloedmonsters. De uitkomsten van deze studie tonen aan dat manipulatie van vers bloed door Ficoll isolatie en cryopreservatie een direct gevolg heeft op de pDC/mDC ratio en de maturatie status van DC. Dit wordt niet veroorzaakt door het gebruik van immunosuppressieve therapie. Deze resultaten laten zien dat bij voorkeur verse bloedmonsters moeten worden gebruikt voor het monitoren van DC.

In deel 2 hebben we het effect van vermindering van de immunosuppressieve medicatie bij ontvangers van een HLA-identieke familieniertransplantaat op de donor-reactieve T-cel respons onderzocht.

Het gebruik van immunosuppressieve geneesmiddelen kan leiden tot zeer ernstige bijwerkingen.[19] Daarom is het wenselijk zo weinig mogelijk immunosuppressie te geven zonder dat dit leidt tot afstoting van het donororgaan. Volgens de literatuur leidt vermindering of stoppen van immunosuppressieve medicatie na orgaantransplantatie tot een toename in donorgerichte T-cel reactiviteit.[20-23] Volledig stoppen van de immunosuppressieve medicatie is slechts beschreven in een klein aantal niertransplantatiepatiënten die lang na transplantatie een stabiele nierfunctie hadden en geen klinische tekenen van afstoting vertoonden.[24-26] Er is echter geen literatuur beschikbaar over de noodzakelijke hoeveelheid immunosuppressieve medicatie bij ontvangers van een HLA-identieke familieniertransplantaat. Tevens is het onbekend of de donorgerichte T-cel reactiviteit wordt beïnvloed na het verminderen van immunosuppressie bij deze patiënten **(Deel II)**. Op onze polikliniek wordt een groep HLA-identieke familieniertransplantatiepatiënten gecontroleerd die azathioprine (AZA) en corticosteroiden als immunosuppressieve onderhoudstherapie gebruikten. Allen waren meer dan 2 jaar na transplantatie. We hebben eerst de AZA dosering verminderd tot 50% van de originele dosering. Bij deze patiënten werd het effect van vermindering van de AZA dosering op de nierfunctie en de T-cel reactiviteit tegen donorcellen onderzocht **(Hoofdstuk 7 en 8)**. De HLA-identieke patiënten bleven vrij van acute afstoting en de nierfunctie bleef stabiel na vermindering van AZA. Zowel voor als na vermindering van AZA bleef het aantal donor-reactieve GrB producerende cellen onveranderd. Opmerkelijk was dat vóór vermindering van de AZA dosering slechts 20% van de patiënten reageerde op donorcellen, terwijl dit na vermindering van de AZA dosering 57% van de patiënten besloeg. Het aantal GrB producerende cellen gericht tegen 3^{e}-partij antigeen nam eveneens toe na vermindering van de AZA dosering **(Hoofdstuk 7)**. Er werd echter geen toename geconstateerd in het aantal donor, 3^{e}-partij en tetanus toxoid (TET)-reactieve IFN-γ en IL-13 producerende cellen **(Hoofdstuk 8)**. Daarnaast vonden we geen relatie tussen het aantal donorreactieve

cytokinen en het aantal bekende mHAg verschillen tussen donor en ontvanger.

Omdat alle HLA-identieke patiënten vrij bleven van acute afstoting, de nierfunctie stabiel bleef en geen toename in donorreactiviteit werd gevonden na vermindering van de AZA dosering, vroegen wij ons af of de immunosuppressieve medicatie nog verder kon worden verminderd **(Hoofdstuk 9 en 10)**. Daarom werden patiënten die tenminste 1 jaar na transplantatie waren eerst op AZA (50 mg/dag) of mycofenolaat mofetil (MMF; 500 mg/dag) therapie gezet in combinatie met 5 mg/dag prednisolon. Het volledig stoppen van AZA of MMF was succesvol bij 85% (23/27) van de patiënten **(Hoofdstuk 9)**. Alle patiënten bleven vrij van acute afstoting en de nierfunctie bleef stabiel, ook na 24 maanden follow-up. Daarnaast lieten andere klinische parameters, zoals bloeddruk, serum lipiden (LDL, HDL en triglyceriden) concentraties, glucose, HbA1c en CRP, geen verandering zien vanaf inclusie tot 24 maanden op monotherapie steroïden. Wel ontwikkelde 15% van de patiënten (4/27) terugkeer van hun oorspronkelijke ziekte. Dit percentage was echter vergelijkbaar met de terugkeer van de oorspronkelijke ziekte uit een andere klinische studie bij HLA-identieke familieniertransplantatiepatiënten die hun hoge dosering immunosuppressie bleven behouden.[27]

In **Hoofdstuk 10** hebben we aangetoond dat het stoppen van AZA of MMF geen significant effect had op de aantallen donor, 3^{e}-partij en TET-reactieve IFN-γ en GrB producerende cellen. Opmerkelijk was dat het aantal donor, 3^{e}-partij en TET-reactieve IL-10 producerende cellen steeg ten tijde van monotherapie steroïden. Blijkbaar onderdrukt AZA en MMF IL-10. AZA en MMF zijn anti-proliferatieve middelen en kunnen ook een effect hebben op de cytokinenproduktie van lymfocyten.[28, 29] Tevens vonden we dat het stoppen van AZA of MMF geen effect had op de absolute aantallen mDC en pDC en de pDC/mDC ratio. De maturatie status van DC subsets was veranderd naar een meer matuur fenotype, hetgeen suggereert dat AZA en MMF de fenotypische maturatie van DC onderdrukt. *In vitro* studies hebben eveneens aangetoond dat AZA en MMF de fenotypische maturatie van DC beïnvloedt.[30-32]

Vervolgens hebben we onderzocht of het stoppen van AZA of MMF een effect had op de donorreactiviteit gemeten in een MLR en cytokinenproductie door middel van de multiplex bead array test **(Hoofdstuk 11)**. Onze resultaten laten zien dat de proliferatieve capaciteit van patiëntencellen gericht tegen donor en 3^{e}-partij antigenen niet werd beïnvloed door AZA of MMF. Uit de multiplex bead array test bleek dat 13 van de 17 donor en 3^{e}-partij reactieve cytokinen konden worden gemeten tijdens combinatietherapie (AZA of MMF + prednisolon) en monotherapie prednisolon. De overige 4 cytokinen waren niet detecteerbaar. Opmerkelijk was dat alleen de productie van donorreactief IFN-γ en MIP-1β hoger was na het stoppen van AZA of MMF, terwijl er geen verschil werd gevonden in de productie van 3^{e}-partij reactieve responsen. Hieruit concluderen we dat donorgerichte reactiviteit kan worden gemeten bij ontvangers van een HLA-identieke familieniertransplantaat met behulp van de multiplex bead array test. AZA en MMF onderdrukken de productie van donorreactief IFN-γ en MIP-1β bij deze patiënten, maar niet de 3^{e}-partij reactiviteit. Tot op heden is de klinische relevantie van deze bevindingen echter nog niet duidelijk.

Gezien de ernstige bijwerkingen van langdurig gebruik van immunosuppressiva en de goede klinische en laboratorium resultaten na afbouw van de AZA en MMF dosering en zelfs het volledig stoppen van AZA of MMF, stellen wij voor de immunosuppressieve medicatie (calcineurin inhibitors [CNI], AZA of MMF) 1 jaar na transplantatie stapsgewijs te stoppen bij HLA-identieke familieniertransplantatiepatiënten. Een lage dosering monotherapie steroïden (5 mg/dag) zou voldoende moeten zijn, op voorwaarde dat de patiënten een stabiele

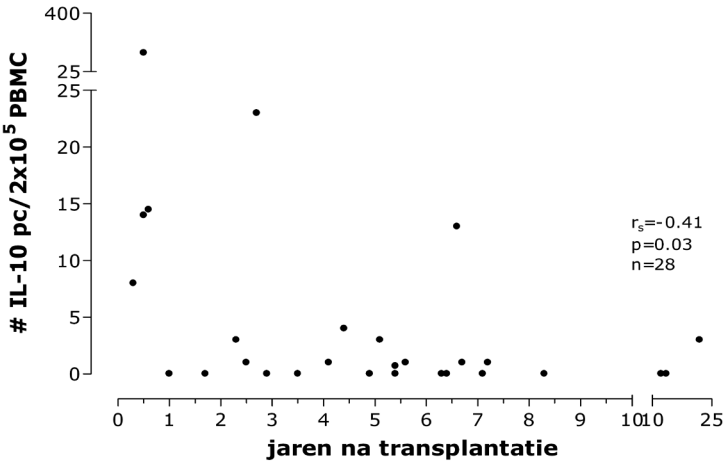
nierfunctie hebben en geen eiwit in de urine vertonen. Hierdoor zullen deze patiënten niet meer langdurig worden blootgesteld aan de ernstige bijwerkingen van immunosuppressiva. Patiënten met een glomerulaire nierziekte als oorspronkelijk lijden, zullen regelmatig poliklinisch moeten worden gecontroleerd om indien nodig de immunosuppressieve therapie aan te passen om terugkeer van de oorspronkelijke ziekte te voorkomen. In de toekomst zou moeten worden onderzocht of het volledig stoppen van de corticosteroïden mogelijk is bij ontvangers van een HLA-identieke familieniertransplantaat. In onze studiepopulatie hebben we inmiddels 2 HLA-identieke patiënten die ook volledig gestopt zijn met hun corticosteroïden medicatie. Deze patiënten zijn nu 3 jaar geheel zonder medicatie, zijn vrij gebleven van afstoting en hebben een stabiele nierfunctie (data niet getoond).

Samenvattend hebben we laten zien dat T-cel reactiviteit tegen donorcellen kunnen worden aangetoond na HLA-identieke familieniertransplantatie. Deze responsen bleken echter niet gerelateerd te zijn aan de bekende mHAg verschillen (HA-1, HA-2, HA-3, HA-8, ACC-1, ACC-2, HwA-9, HwA-10, UGT2B17 en HY) **(Hoofdstuk 3, 4, 8, 10, 11)**. Deze resultaten komen overeen met een studie van Heinold *et al.*[33] die laat zien dat bekende mHAg verschillen geen invloed hebben op de 5-jaars transplantaatoverleving na het ontvangen van een overleden of een levende familieniertransplantatie. Andere studies laten een associatie zien met mHAg verschillen en acute afstoting, chronische afstoting en transplantaatfalen.[34-37] Zij analyseerden deze associaties bij patiënten die een niertransplantaat ontvingen van een overleden donor en niet bij HLA-identieke koppels. Daarom kan er niet worden uitgesloten dat de gevonden associatie werd veroorzaakt door HLA verschillen, zoals HLA-Cw, HLA-DQ en HLA-DP, in plaats van mHAg verschillen tussen donor en ontvanger.[38-41]

In de literatuur is beschreven dat pro-inflammatoire cytokinen, zoals IFN-γ, IL-2 en TNF-α, afstoting van het getransplanteerde orgaan bevorderen, terwijl anti-inflammatoire cytokinen, IL-4, IL-5, IL-10 en IL-13, met name geassocieerd zijn met het onderdrukken van de immuunreactiviteit.[42-44] In onze studies konden met behulp van de Elispot test, tijdens immunologische rust, donorgerichte T-cel reactiviteit worden gedetecteerd in PBMC van HLA-identieke familieniertransplantatiepatiënten **(Hoofdstuk 3, 4, 7, 8, 10)**. Opmerkelijk was dat er een negatieve correlatie (r_s=-0.41; p=0.03) werd gevonden tussen het aantal donorreactieve IL-10 producerende cellen en tijd na HLA-identieke familieniertransplantatie. Dit was gemeten voor vermindering van immunosuppressie (Figuur 1). Voor IFN-γ en GrB werd dit niet gevonden (data niet getoond). Daarna resulteerde het stoppen van AZA of MMF bij deze patiënten in een toename van het aantal donorreactieve IL-10 producerende cellen **(Hoofdstuk 10)**. Deze resultaten suggereren dat er immuunreactiviteit aanwezig is gericht tegen donorantigenen tijdens het eerste jaar na transplantatie en tijdens het stoppen van immunosuppressiva. Dit zou kunnen betekenen dat de aanwezige immuunreactiviteit actief wordt onderdrukt door donorreactief IL-10 bij HLA-identieke familieniertransplantatiepatiënten. Een aantal andere studies heeft ook donorreactief IL-10 bij ontvangers van een HLA-identieke familieniertransplantaat aangetoond, dat mogelijk tot tolerantie kan leiden.[11, 12, 45]

Het grote pluspunt van onze studies is dat geen van de HLA-identieke patiënten een acute afstoting heeft doorgemaakt na het stoppen van AZA en MMF therapie. Daarom hebben we de klinische relevantie van de Elispot test in het voorspellen van acute afstoting niet kunnen aantonen voor deze specifieke patiëntengroep.

Concluderend kunnen we zeggen dat in het perifere bloed van ontvangers van een HLA-identieke familieniertransplantaat T-cel reactiviteit tegen donorcellen kan worden aangetoond. Deze reactiviteit kan niet worden gerelateerd aan het aantal bekende mHAg verschillen tussen donor en ontvanger. Hoge aantallen donorreactieve IL-10 producerende cellen suggereert een actieve onderdrukking van immuunreactiviteit, gericht tegen mHAgs en andere niet-HLA antigenen. Bij deze patiënten kunnen de CNI, MMF en AZA doseringen stapsgewijs 1 jaar na transplantatie veilig worden stopgezet; een lage dosering corticosteroïden blijkt voldoende voor een stabiele nierfunctie.



Figuur 1: Het aantal donorreactieve IL-10 producerende cellen (pc) in PBMC van HLA-identieke familieniertransplantatiepatiënten in relatie tot de tijd na transplantatie voor afbouw van de immunosuppressieve medicatie.

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Abbreviations

Dankwoord

Curriculum Vitae

PhD portfolio

APC	antigen-presenting cells
AZA	azathioprine
BDCA	blood dendritic cells antigens
BMT	bone marrow transplantation
CAN	chronic allograft nephropathy
CKR	chemokine receptors
CNI	calcineurin inhibitor
CRYO	cryopreservation
CsA	cyclosporine A
CTL	cytotoxic T lymphocyte
CTLpf	cytotoxic T-lymphocyte precursor frequency
DC	dendritic cells
FB	fresh blood
GrB	granzyme B
GVHD	graft-*versus*-hose disease
HF	heart failure
HLA	human leucocyte antigens
HTLpf	helper T-lymphocyte precursor frequency
IFN	interferon
IL	interleukin
KTx	kidney transplantation
LDA	limiting dilution assay
LR	living-related
mAbs	monoclonal antibodies
mDC	myeloid DC
mHAgs	minor histocompatibility antigens
MHC	major histocompatibility antigens
MIP-1β	macrophage inflammatory protein-1β
MLR	mixed lymphocyte reaction
MMF	mycophenolate mofetil
mo	month
NFAT	nuclear factor of activated T cells
PBMC	peripheral blood mononuclear cells
pc	producing cells
PCR	polymerase chain reaction
pDC	plasmacytoid DC
PHA	phytohemagglutinin
Pred	predniso(lo)n(e)
RTx	renal transplantation
SI	stimulation index
Tacro	tacrolimus
TET	tetanus toxoid
Th	T helper cells
TNF	tumor necrosis factor
Tr1	Type-1 regulatory T cells
Tregs	regulatory T cells

Dankwoord

‘Altijd als je denkt: Dit is het einde
sta je op de grens van wat begint.
Altijd als je alles denkt te kennen,
Laat alles zich opeens weer anders zien.’
(Stef Bos: ‘Duizend jaar’)

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Jeroen H. Gerrits

Curriculum Vitae

Jeroen Hendrik Gerrits was born on September 26th, 1977 in Hardenberg, the Netherlands. From 1989 to 1993, he attended Lower General Secondary Education (MAVO) at the Vechtstede in Hardenberg. In September of that year he started the College of Laboratory (MLO; Clinical Chemistry) at the Drenthe College in Emmen and graduated in 1997. Thereafter, he attended the Higher Laboratory School (HLO; Biomedical Chemistry) at the Saxion Hogeschool in Deventer. His practical training took place at the Department of Bloodtransfusion and Transplantation Immunology of the University Medical Center St. Radboud Nijmegen under supervision of Dr. A. van der Meer. From 2000-2003, he studied Biology (Medical Biology) at the Radboud University Nijmegen. Undergraduate research was conducted at the Department of Cell Biology under supervision of Drs. Ing. S.P. van der Woning and Prof. Dr. E.J. van Zoelen of the Radboud University Nijmegen, and at the Department of Central Haematology Laboratory under supervision of Dr. H. Dolstra of the University Medical Center St. Radboud Nijmegen. In September 2003, he started his PhD research at the Department of Internal Medicine, section Transplantation of the Erasmus MC in Rotterdam under supervision of Dr. N.M. van Besouw and Prof. Dr. W. Weimar. The results of the study 'Donor-Reactive T-cell Responses after HLA-Identical Living-Related Kidney Transplantation' are described in this thesis. Since July 2009 he is working at LabNoord as Clinical Chemist trainee. He lives with Sandra Mulder and their son Lars.

PhD Portfolio

Name PhD student:	Jeroen Hendrik Gerrits
Erasmus MC Department:	Internal Medicine-Transplantation
Research School:	MolMed
PhD period:	Sept 2003 – June 2008
Promotor:	Prof. Dr. W. Weimar
Co-promotor:	Dr. N.M. van Besouw

1. PhD training

General academic skills

- Biomedical English Writing and Communication course	2005
- Basic teaching course	2006
- International Conference on Harmonisation of Good Clinical Practice (ICH-GCP)	2007

Research skills

- Statistics: Classical Methods for Data analysis (Nihes)	2004

In-depth courses (e.g. Research school, Medical Training)

- Molecular Immunology (Postgraduate School of Molecular Medicine)	2005

(Inter)national conferences

– Annual Meeting NVVI[1], Noordwijkerhout, The Netherlands	Dec 18-19,	2003
– Annual Meeting NTV[2], Texel, the Netherlands	March 17-19,	2004
– Annual Meeting Dutch Society of Nephrology, Veldhoven, the Netherlands	March 25-26,	2004
– International Transplantation Society Congress, Vienna, Austria	Sept 5-10,	2004
– Annual Meeting NTV, Kerkrade, the Netherlands	March 9-11,	2005
– Annual Meeting Dutch Society of Nephrology, Veldhoven, The Netherlands	April 5-6,	2005
– American Transplant Congress, Seattle, USA	May 21-25,	2005
– European Society for Organ Transplantation Congress, Geneva, Switzerland	Oct 16-19,	2005
– Annual Meeting NVVI, Noordwijkerhout, The Netherlands	Dec 8-9,	2005
– Annual Meeting NTV, Zeewolde, The Netherlands	March 15-17,	2006
– World Transplant Congress, Boston, USA	July 22-27,	2006
– Annual Meeting NVVI, Noordwijkerhout, The Netherlands	Dec 7-8,	2006
– Annual Meeting NTV, Zeewolde, The Netherlands	March 28-30,	2007
– Annual Meeting Dutch Society of Nephrology, Veldhoven, The Netherlands	April 3-4,	2007
– European Society for Organ Transplantation Congress, Prague, Czech Republic	Sept 30-Oct 3,	2007
– Annual Meeting Department of Internal Medicine Erasmus MC, Antwerp, Belgium	Jan 10-11,	2008

[1]NVVI, Nederlandse Vereniging voor Immunologie (Dutch Society for Immunology)
[2]NTV, Nederlandse Transplantatie Vereniging (Dutch Transplantation Society)

Presentations

– Overimmunosuppression after HLA-identical living-related kidney transplantation. Annual Meeting Dutch Society of Nephrology, 2004 (Award for best abstract of session 'Scientific Session IV')	Oral
– Overimmunosuppression after HLA-identical living-related kidney transplantation. International Transplantation Society Congress, 2004	Poster
– Stable donor-specific reactivity during tapering of immunosuppression after HLA-identical living-related kidney transplantation. Annual Meeting NTV, 2005	Oral
– Stable donor-specific reactivity during tapering of immunosuppression after HLA-identical living-related kidney transplantation. Annual Meeting Dutch Society of Nephrology, 2005	Oral
– Stable donor-specific T-cell reactivity during tapering of azathioprine after HLA-identical living-related kidney transplantation. American Transplant Congress, 2005	Poster

– High frequencies of donor-specific IL-10 producing cells after HLA-identical living-related kidney transplantation. European Society for Organ Transplantation Congress, 2005	oral
– Cytokine-profiles after HLA-mismatched and HLA-identical living-related kidney transplantation. Annual Meeting NVVI, 2005	poster
– T-cell reactivity during monotherapy of predisone after HLA-identical living-related kidney transplantation. Molecular Medicine day, 2006	oral
– T-cell reactivity during monotherapy prednisone after HLA-identical living-related kidney transplantation. Annual Meeting NTV, 2006 (Award for best presentation of session 'Plenary session II)	oral
– Cytokine-profiles after HLA-mismatched and HLA-identical living-related kidney transplantation. Annual Meeting NTV, 2006	oral
– T-cell reactivity during monotherapy prednisone after HLA-identical living-related kidney transplantation. World Transplant Congress, 2006	poster
– Cytokine-profiles after HLA-mismatched and HLA-identical living-related kidney transplantation. World Transplant Congress, 2006	poster
– T-cell reactivity during prednisone monotherapy after HLA-identical living-related kidney transplantation. Annual Meeting NVVI, 2006	poster
– Donor-specific T-cell reactivity during steroid monotherapy after HLA-identical living-related kidney transplantation. Annual Meeting NTV, 2007	oral
– Donor-specific T-cell reactivity during steroid monotherapy after HLA-identical living-related kidney transplantation. Annual Meeting Dutch Society of Nephrology, 2007	oral
– Donor-specific T-cell reactivity during steroid monotherapy after HLA-identical living-related kidney transplantation. American Transplant Congress, 2007	poster
– T-cell reactivity in HLA-identical living-related kidney transplantation during steroid monotherapy. European Society for Organ Transplantation Congress, 2007	oral
– T-cell reactivity in HLA-identical living-related kidney transplantation during steroid monotherapy. Annual Meeting Internal Medicine, 2008	oral
– Donor-reactive T-cell responses even after HLA-identical living-related kidney transplantation. American Transplant Congress 2008	poster

Seminars and workshops

- 'Winterschool' Dutch Kidney Foundation	Jan 7-10, 2004
- Clinical Review Symposium NTV, Utrecht, the Netherlands	Nov 25, 2004
- Seminar 'New developments in medical immunology', Rotterdam, the Netherlands	Nov 23, 2005
- 10th Molecular Medicine Day, Rotterdam, the Netherlands	Febr 1, 2006
- Clinical Review Symposium NTV, Utrecht, the Netherlands	Nov 23, 2006

2. Teaching activities

Lecturing

- Lecture for PhD students/Medical Doctors (ESOT course)	Nov 29, 2004
- Lecture for PhD students/Medical Doctors (ESOT course)	Oct 30, 2006

Supervising practicals

- 1st ESOT Hands-on-Course Laboratory Techniques	Nov 29-Dec 1, 2004
- 2nd ESOT Hands-on-Course Laboratory Techniques	Oct 30-Nov 2, 2006