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ORIGINAL ARTICLE

Impact of maintenance immunosuppressive regimens – balance between graft protective suppression of immune functions and a near physiological immune response

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Summary

The Symphony study showed superior 1-year kidney graft outcome in patients on immunosuppression with tacrolimus/mycophenolate mofetil (Tacr/MMF). To analyze whether differences in clinical outcome between maintenance regimens may be explained by their impact on clinically relevant immune parameters, we assessed CD4 helper activity, immunoglobulin-secreting cell (ISC) formation, neopterin, sCD30, and intracellular cytokine production in a prospective study in 77 renal transplant recipients treated with cyclosporine A/azathioprine (CsA/Aza), CsA/MMF, Tacr/Aza or Tacr/MMF at 2 years post-transplant. Tacr- compared with CsA-based immunosuppression was independently associated with increased IL-2 ($P < 0.0001$, CD4 cells; $P = 0.014$, CD8 cells) and CD4 cell IL-4 responses ($P = 0.046$; stepwise logistic regression) resulting in physiological responses in Tacr/Aza patients as compared with 25 healthy controls. MMF versus Aza treatment was proven to be an independent variable associated with suppression of CD4 cell IL-10 responses ($P = 0.008$), B-cell IL-6R expression ($P < 0.0001$) and ISC formation [$P = 0.020$, staphylococcus cowan strain I (SAC I); $P = 0.021$, pokeweed mitogen (PWM)]. Our data suggest that Tacr/MMF had the most effective impact on graft protective Th2 responses (enhanced CD4 cell IL-4 by Tacr, decreased CD4 cell IL-10 responses by MMF) and suppression of B-cell functions (MMF), whereas Tacr/Aza was associated with physiological IL-2 and IL-4 and stronger humoral responses which may reduce the risk of infectious disease complications.

(ClinicalTrials.gov number: NCT00150891).

Introduction

The Symphony study [1] found that 1-year graft outcome – acute rejection incidence, graft function and survival – was best in tacrolimus/mycophenolate mofetil (Tacr/MMF)-treated patients. We hypothesized that this superior clinical outcome might be related to a more effective impact of this regimen on clinically relevant immune parameters. It must be considered that the most potent immunosuppressive regimen may not be the best for all patients, as increased side effects such as post-transplant *de novo* diabetes [2], polyomavirus-associated nephropathy [3], infectious disease [4], or malignancy [5–8] have a negative impact that counteracts benefits in terms of graft and patient survival. Thus, patient-tailored immunosuppressive therapy, which encompasses consideration of underlying clinical risks of

the recipient, side effects of immunosuppressive drugs as well as individual immunologic risk profiles, may offer optimal benefits for the patient.

In vitro, Th2, B-cell and monocyte responses were shown to predict acute and chronic graft rejection and may therefore serve as parameters for selecting patient-tailored immunosuppressive regimens. We found previously that pretransplant **Th2 responses** (low CD4 helper activity and CD4 cell IL-10 responses, enhanced CD4 cell IL-4 responses) were associated with a low risk of acute rejection and improved 1- and 3-year graft function [9–11]. Long-term stable renal allograft recipients showed diminished T helper and elevated T suppressor functions compared with patients with chronic graft dysfunction [12]. Süsal *et al.* found that pretransplant high serum soluble CD30 (sCD30), as a costimulatory molecule in the regulation between Th1 and Th2 responses [13], was associated with impaired kidney graft survival [14,15]. With respect to **B-cell responses**, we showed that elevated pretransplant PWM- or SAC I-stimulated immunoglobulin-secreting cell (ISC) formation defined patients at high risk of early acute rejection [16,17], whereas long-term stable renal allograft recipients demonstrated diminished T-dependent B-cell responses, together with reduced monocyte activity [18]. There is growing evidence that post-transplant B-cell responses resulting in the formation of donor-specific HLA antibodies play an important role in the development of chronic allograft dysfunction [19–23]. **Monocytes and macrophages** also play an important role in chronic rejection [24–26]; as a corollary, we found 1-year neopterin plasma levels together with 1-year sCD30 to be predictive of chronic graft dysfunction [27].

In the present study, we analyzed the impact of immunosuppressive drugs on immunological parameters of graft outcome as a basis for selection of an appropriate recipient-tailored immunosuppressive regimen. Eighty-four renal transplant recipients were initially enrolled in a prospective study, randomized to receive CsA/Aza, CsA/MMF or Tacr/Aza. Effects of the immunosuppressive maintenance treatment (CsA/Aza, CsA/MMF, Tacr/Aza, Tacr/MMF) on Th1/Th2, B-cell and monocyte responses were analyzed at 2 years post-transplant; this largely rules out effects of acute rejection, CMV activation and switches of immunosuppressive regimens, which nearly all occurred during the first 18 months post-transplant.

Materials and methods

Patients and controls

Eighty-four recipients of 63 deceased and 21 living donor renal allografts who were transplanted at the Giessen transplant center, were prospectively randomized to one of three immunosuppressive regimens: CsA/Aza, CsA/MMF, or Tacr/Aza [11,27]. All patients received in addition prednisolone, and tapering was started not earlier than 6 months post-transplant. Prophylactic rabbit ATG (4 mg/kg/day; Fresenius, Oberursel, Germany) induction therapy was administered in patients with an increased immunological risk profile (retransplants, panel reactive antibody (PRA) >5%, or post-transplant acute renal failure). Monoclonal anti-CD25 antibodies were not used. The three patient groups were comparable with respect to clinical features as described previously [27].

Four early graft losses occurred because of acute humoral rejection ($n = 3$) and venous graft thrombosis ($n = 1$) in Tacr/Aza patients and two further graft losses because of interstitial fibrosis/tubulus atrophy (IF/TA) in CsA/MMF patients and therefore immunological tests could be carried out in 77 of the 84 patients at 2 years post-transplant (one patient on sirolimus was not included). Switching of the immunosuppressive regimens is shown in Table 1. Most switchings (28/37, 76%) took place during the first post-transplant year, and 36/37 (97%) took place during the first 18 months.

Twenty-two of all 25 (88%) acute rejection episodes observed during the first 2 years occurred within the first post-transplant year, and 15 of 25 (60%) were confirmed by renal biopsy. Chronic allograft dysfunction (eight patients with functioning grafts included in the 2-year analysis) was defined clinically by progressive decrease of graft function, exclusion of renal artery stenosis, and confirmation of IF/TA by renal biopsy.

Twenty-five healthy blood donors, whose age distribution was comparable with the patients', were tested as controls for intracellular cytokine production, expression of cytokine receptors and costimulatory ligands.

Flow cytometric analysis

Peripheral blood mononuclear cell (PBMC) subsets and their expression of cytokine receptors and costimulatory ligands were determined by double-fluorescence laser flow-cytometry as described previously [9,28]. The following monoclonal antibodies (MoAb) were used: OKT3-FITC (CD3; Ortho, Neckargemünd, Germany), anti-CD4-FITC, anti-CD4-PE, anti-CD8-FITC, anti-CD14-FITC, anti-CD19-FITC, anti-CD25-PE, anti-CD28-PE, anti-CD40-PE, anti-CD56-FITC, anti-B7-1-PE (CD80) and anti-B7-2-PE (CD86), anti-IL-10R-PE, anti-CD154-PE [Becton Dickinson (BD), Heidelberg, Germany] and anti-CD126-PE (IL-6R; Coulter Immunotech, Krefeld, Germany). Measurements were performed using a Cytoron flow cytometer (Ortho) for absolute cell count determination, and a FACSCalibur flow cytometer (BD) for double fluorescence analysis as described under ''intracellular cytokine determination''.

Table 1. Transplant data and 2-year outcome of patients who were treated with cyclosporine A/azathioprine (CsA/Aza), cyclosporine A/mycophenolate mofetil (CsA/MMF), tacrolimus/azathioprine (Tacr/Aza) and Tacr/MMF, respectively, at the 2-year post-transplant time point.

	CsA/Aza	CsA/MMF	Tacr/Aza	Tacr/MMF	*P**
Number of patients	16	22	27	12	
Recipient age (years)	52 ± 3	46 ± 3	47 ± 3	39 ± 3	0.077
Waiting time (months)	52 ± 8	46 ± 7	56 ± 8	39 ± 15	0.195
Living donors†	19% (3)	18% (4)	22% (6)	58% (7)	0.072
Number of retransplants	13% (2)	14% (3)	15% (4)	17% (2)	1.000
PRA max‡	6 ± 1%	8 ± 3%	9 ± 2%	5 ± 1%	0.821
Blood transfusions	3.1 ± 1.1	5.4 ± 3.6	3.0 ± 1.3	5.0 ± 2.0	0.559
Pregnancies	38% (6)	27% (6)	19% (5)	33% (4)	0.523
Donor age (years)	46 ± 5	49 ± 2	47 ± 3	48 ± 4	0.982
HLA-A,B,DR mismatches	2.1 ± 0.4	2.9 ± 0.4	2.3 ± 0.3	2.3 ± 0.4	0.651
HLA-B,DR mismatches	1.4 ± 0.3	2.1 ± 0.3	1.7 ± 0.2	1.5 ± 0.3	0.263
ATG induction therapy	56% (9)	55% (12)	52% (14)	33% (4)	0.630
Initial immunosuppressive regimens§					
CsA/Aza	75% (12/16)	14% (3/22)	26% (7/27)	17% (2/12)	
CsA/MMF	13% (2/16)	86% (19/22)	7% (2/27)	50% (6/12)	
Tacr/Aza	13% (2/16)	0% (0/22)	67% (18/27)	33% (4/12)	
CsA trough level (ng/ml)	170 ± 17	206 ± 14			0.188
Tacr trough level (ng/ml)			7.9 ± 0.5	8.9 ± 0.6	0.210
MPA trough level (μg/ml)		2.8 ± 0.5		3.0 ± 0.5	0.542
Prednisolone (mg/day)¶	4.4 ± 1.0	4.0 ± 0.8	2.6 ± 0.6	5.5 ± 2.0	0.368
Steroid free¶	25% (4)	27% (6)	37% (10)	42% (5)	0.712
AR incidence**	6% (1)	14% (3)	26% (7)	58% (7)	0.011
Late AR incidence**	0% (0)	5% (1)	7% (2)	25% (3)	0.117
AMR incidence**	0% (0)	5% (1)	0% (0)	8% (1)	0.298
Graft function††					
S-Cr (mg/dl)††	2.4 ± 0.3	2.3 ± 0.1	1.8 ± 0.1	1.9 ± 0.2	0.172
ClCr (ml/min)††	43 ± 7	43 ± 4	60 ± 7	61 ± 9	0.060
Severe infectious disease‡‡	50% (8)	32% (7)	30% (8)	17% (2)	0.315
CMV disease§§	50% (8)	46% (10)	26% (7)	33% (4)	0.357
BK nephropathy	6% (1)	0% (0)	0% (0)	0% (0)	0.364
IF/TA¶¶	6% (1)	18% (4)	0% (0)	8% (1)	0.089

Seventy-eight patients had functioning grafts two years post-transplant. One patient on sirolimus therapy is not included in this study.

CsA, cyclosporine A; Aza, azathioprine; MMF, mycophenolate mofetil; MPA, mycophenolic acid; Tacr, tacrolimus.

*Kruskall–Wallis H test, Chi-square test and Fisher's exact test, respectively, were used for statistical comparison of the four patient groups.

†Percentage of patients receiving transplants from living related or unrelated donors.

‡PRAmax = Maximum panel reactive antibodies pretransplant; with respect to recent pretransplant PRA (data not shown) no significant differences were found either ($P = 0.317$).

§Within the first two years post-transplant, 19/56 (34%) patients were switched from CsA to Tacr because of acute rejection ($n = 12$), gum hyperplasia and/or hirsutism ($n = 5$), or neurotoxicity ($n = 2$). Conversely, 2/28 (7%) patients were switched from Tacr to CsA because of BK nephropathy and hepatotoxicity, respectively. MMF was stopped in 5/31 (16%) patients because of side effects ($n = 4$) or non-compliance ($n = 1$). Aza was withdrawn in 19/53 (36%) patients because of side effects ($n = 7$), breast cancer ($n = 1$), acute rejections ($n = 8$), or chronic graft dysfunction ($n = 2$). Switches were more frequent in CsA/Aza patients [16/25 (64%) of CsA/Aza patients were switched vs. 12/31 (39%) of CsA/MMF and 10/28 (36%) of Tacr/Aza patients], but the differences did not reach statistical significance ($P = 0.078$). Most switchings (28/37, 76%) took place during the first post-transplant year, and 36/37 (97%) took place during the first 18 months. In this Table, only switchings of the 77 patients included in the 2-year study population are shown.

¶Daily prednisolone dosage and percentage of patients who were off steroids at the 2-year post-transplant time point.

**Acute rejection (AR) and acute antibody-mediated rejection (AMR) incidence; late AR (between months 5 and 24).

††Two-year graft function is given by serum creatinine (S-Cr) and measured creatinine clearance (ClCr).

‡‡Severe infectious disease was defined as need for in-hospital treatment.

§§CMV disease (CMV syndrome included) occurred mainly within the first 4 months post-transplant (30 patients versus 4 patients between 5 and 24 months post-transplant).

¶¶Graft deterioration as a result of interstial fibrosis/tubulus atrophy (IF/TA) as confirmed by graft biopsy.

Intracellular cytokine determination

Peripheral blood mononuclear cells were isolated from heparinized blood by Ficoll gradient centrifugation within 3 h after blood withdrawal. PBMC at 10^6/ml were cultured in RPMI with glutamine (Invitrogen, Karlsruhe, Germany) supplemented with 10% human AB serum, 1 mM natrium pyruvate, 5 μM beta-mercaptoethanol and penicillin–streptomycin. PBMC were cultured at 37 °C in a 5% CO_2 humidified atmosphere, either alone or in the presence of 10 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma, Taufkirchen, Germany) and 0.75 μg/ml ionomycin (Sigma) in 50 ml Costar culture vials (Corning, Wiesbaden, Germany) for 4 h and alone or in the presence of lipopolysaccharide (LPS, 100 ng/ml; Merck, Darmstadt, Germany) for 24 h in glass test tubes, respectively. Brefeldin A (Golgi-Plug®; BD) was added for the last 4 h of the culture period to induce accumulation of secretory proteins in the Golgi stacks. Cells were washed and incubated with fixation and permeabilizing solution (Cytofix/Cytoperm®; BD) for 20 min at 4 °C. Cells were washed (Perm/Wash®; BD) and stained with fluorescent-conjugated anticytokine antibodies as given in Table 2. Measurements were performed using a FACSCalibur flow cytometer. Exclusion of dead cells was done by forward- and side-scatter gating. One hundred thousand mononuclear cells were typically acquired for analysis of intracellular cytokines and surface markers, respectively. Results are given in percentage of gated cells and mean fluorescence intensity (MFI) for indicated parameters (those showing better discrimination from isotype controls by MFI), respectively. The results were calculated by subtracting percentage of gated cells and mean channel value, respectively, of the respective isotype controls.

Table 2. Intracellular cytokine determination of peripheral blood mononuclear cells (PBMC) stimulated with PMA/ionomycin and LPS, respectively, using flow cytometry and triple fluorescence analysis.

MoAb* combinations used for staining of cultured PBMC			
PMA/ionomycin-stimulated cultures			
Anti-CD3-PerCP/Anti-CD69-PE			
Anti-CD3-PerCP/Anti-CD8-FITC†/Anti-IL-2-PE			
Anti-CD3-PerCP/Anti-CD8-FITC†/Anti-IL-4-PE			
Anti-CD3-PerCP/Anti-CD8-FITC†/Anti-IL-10-PE			
Anti-CD19-PerCP/Anti-IL-6-FITC/Anti-IL-10-PE			
LPS-stimulated cultures			
Anti-CD19-PerCP/Anti-CD14-FITC/Anti-IL-6-PE‡			
Anti-CD19-PerCP/Anti-CD14-FITC/Anti-IL-10-PE‡			
Isotype controls: mouse IgG1-FITC and IgG1-PE, rat IgG1-PE			
Cell viability§			
PMA/ionomycin-stimulated cultures:			
CD4+ T cells: 99.4 ± 0.2%; CD8+ T cells: 98.4 ± 1.4%; B cells: 99.5 ± 0.1%			
LPS-stimulated cultures:			
Monocytes: 97.2 ± 1.9%			
Reliability (Mean SD of duplicate measurements)¶			
CD69+ T cells 0.7%			
CD4 cell IL-2 2.2%	CD8 cell IL-2 1.0%	B-cell IL-6 3.1%	Monocyte IL-6 4.3%
CD4 cell IL-4 0.3%	CD8 cell IL-4 0.3%	B-cell IL-10 10.8**	Monocyte IL-10 15.7**
CD4 cell IL-10 6.2**	CD8 cell IL-10 7.1**		** = MFI

PBMC, peripheral blood mononuclear cell.

*All MoAb (trade names as given in the Table) were purchased from BD. For intracellular cytokine analysis of B cells, staining with anti-CD19-PerCP had to be performed before fixation and permeabilization with Cytofix/Cytoperm® and staining with anti-cytokine antibodies. Before staining of monocytes, cells were incubated for 10min with rat IgG Fc fragments (FC-Block®, Dianova, Hamburg, Germany) to prevent unspecific staining of monocytes by anticytokine antibodies. In each experiment, isotype controls and CD69 activation control were run concomitantly.

†Because of downregulation of the CD4 molecule as early as 4h after PMA/ionomycin stimulation, CD4+ T cells were assumed to be those CD3+ T cells which were CD8-.

‡In LPS-stimulated cultures, anti-CD19-PerCP MoAb was not used for assessment of cytokine production in B cells, but to enhance validity of intracellular monokine determination: CD14 and CD19 double positive cells were excluded from monokine assessment, as double positive cells are a result of autofluorescence and especially unspecific antibody binding by Fc receptors.

§Exclusion of dead cells was routinely done by forward- and side-scatter gating. Within the gates adjusted to assess cytokine production of different mononuclear subsets, viability was tested by 7-amino-actinomycin D (7-AAD; BD) staining. Results, given as mean and standard deviation (SD), are indicated.

¶Inter-assay variability was calculated from duplicate cultures in five healthy controls. Mean SD values of the tested parameters are indicated, expressed in % of gated cells and mean fluorescence intensity (MFI), respectively.

Serum sCD30 and neopterin

Sera were thawed and tested by ELISA for sCD30 (Bender MedSystems, Vienna, Austria) and neopterin (Brahms, Berlin, Germany). Neopterin values were corrected for graft function by dividing values by the serum creatinine (Neopterin/S-Cr, given in nmol/mg S-Cr).

IgG antibodies against HLA class I and II antigens

IgG-anti-HLA class I and II antibodies were assessed pre-transplant and 2 years post-transplant as described previously [27].

PWM-stimulated allogeneic cocultures and SAC I-stimulated B-cell cultures

B-cell responses of PWM-stimulated allogeneic cocultures of patient B and control T cells, and of SAC I-stimulated B-cell cultures, and CD4+ T-cell helper activity were determined as described previously [9,18,29]. Formation of immunoglobulin-secreting cells (ISC) was assessed in a reverse hemolytic plaque assay. Helper activity of CD4+ T cells and B cell responses were calculated from the results (ISC/10^6 B cells) of the following cocultures:

CD4 cell helper activity:

$$\frac{(\mathrm{B(C)} + \mathrm{T4(P)} + \mathrm{PWM}) - (\mathrm{B(C)} + \mathrm{T4(P)} + \mathrm{M})}{(\mathrm{B(C)} + \mathrm{T(C)} + \mathrm{PWM}) - (\mathrm{B(C)} + \mathrm{T(C)} + \mathrm{M})}$$

PWM-stimulated B cell response:

$$\frac{(\mathrm{B(P)} + \mathrm{T(C)} + \mathrm{PWM}) - (\mathrm{B(P)} + \mathrm{T(C)} + \mathrm{M})}{(\mathrm{B(C)} + \mathrm{T(C)} + \mathrm{PWM}) - (\mathrm{B(C)} + \mathrm{T(C)} + \mathrm{PWM})}$$

SAC I-stimulated B cell response:

$$(\mathrm{B(P)} + \mathrm{SAC\ I}) - (\mathrm{B(P)} + \mathrm{M})$$

B(C), T(C) = B and T cells of a control; B(P), T(P), T4(P) = patient B or T cells or CD4+ T cells. M = culture medium.

Statistics

Observed data are presented as mean and SEM. Wilcoxon rank-sum test, Wilcoxon signed-rank test, Kruskall–Wallis H test, pairwise comparison holding experiment-wise error as a *post hoc* test correcting for multiple testing, Chi-square or Fisher's exact test were used for statistical analysis. Stepwise logistic regression analysis was performed as described in Table 4.

Results

Transplant outcome

Although this study deals with immunological effects related to the immunosuppressive treatment at 2 years post-transplant, we will first provide an intention-to-treat analysis of transplant outcomes of our prospective study. The three patient groups randomized pretransplant to receive CsA/Aza, CsA/MMF or Tacr/Aza, respectively, showed no significant differences in 2-year patient or graft survival, nor in the incidence of acute rejection, CMV disease, severe infectious disease or chronic allograft dysfunction (data not shown). Two-year graft function as determined by measured creatinine clearance was significantly different among the three treatment groups (65 ± 8 ml/min, Tacr/Aza; 42 ± 5 ml/min, CsA/Aza; 48 ± 4 ml/min, CsA/MMF; $P = 0.015$) and Tacr compared with CsA treatment proved to be an independent variable associated with a better 2-year graft function [$P = 0.014$, stepwise logistic regression (lreg)].

In the main component of this study, we analyzed immunological effects in relation to the immunosuppressive maintenance treatment administered at 2 years post-transplant in four immunosuppressive treatment groups (CsA/Aza, CsA/MMF, Tacr/Aza, Tacr/MMF; Table 1). As we found differences among the groups concerning the percentage of living donors, acute rejection incidence, recipient age and graft function/creatinine clearance (Table 1), these parameters were appropriately considered in multivariate analysis. Patients on Tacr/MMF at 2 years, a majority of whom were switched from other regimens because the patients experienced acute rejection, naturally showed the expected increased incidence of previous acute rejection (Table 1) and therefore comprised a group of increased immunological risk.

Impact of immunosuppression on T and NK cells

Flow cytometry

Tacrolimus/MMF patients showed downregulated CD25 expression on CD8 cells and downregulated CD28 expression on CD4 cells (Tables 3 and 4). However, multivariate analysis demonstrated that these effects were primarily related to an increased acute rejection incidence or the coinciding rejection treatment (methylprednisolone pulse; $P = 0.044$ and $P = 0.013$, respectively; lreg) found in the Tacr/MMF treatment group.

Compared with healthy controls, CD25 expression was profoundly upregulated on CD4+ T cells ($P = 0.005$ for all patients; Table 3). CD28 expression was significantly suppressed on patients' CD8+ T cells ($P < 0.001$), whereas CD154 expression was strongly upregulated on the patients' CD4+ T cells ($P = 0.007$; Table 3).

Interestingly, NK cell concentrations were significantly enhanced in CsA/MMF-treated patients compared with the other patient groups ($P = 0.001$; Table 3), which might decrease the risk of infectious complications.

Table 3. Peripheral blood mononuclear cell (PBMC) concentrations, their intracellular cytokine production and expression of cytokine receptors and costimulatory ligands, helper activity of CD4+ T cells, sCD30 and neopterin in the four patient groups treated with CsA/Aza, CsA/MMF, Tacr/Aza and Tacr/MMF, respectively, at 2 years post-transplant. In addition, intracellular cytokine production and PBMC expression of cytokine receptors and costimulatory ligands in 25 healthy controls were compared with the whole patient group and with each of the four patient groups, respectively.

	Controls	Patients	*P** controls versus patients	CsA/Aza	*P*† controls versus CsA/Aza	CsA/MMF	*P*† controls versus CsA/MMF	Tacr/Aza	*P*† controls versus Tacr/Aza	Tacr/MMF	*P*† controls versus Tacr/MMF	*P*‡ patient groups
Number of persons	25	77		16		22		27		12		
Mononuclear cells/μl	ND			2298 ± 271		3316 ± 372		1818 ± 155		1795 ± 261		0.005
T cells/μl	ND			1305 ± 171		1877 ± 303		1003 ± 108		983 ± 167		0.062
CD4/CD8 ratio	ND			1.1 ± 0.2		1.3 ± 0.2		1.6 ± 0.2		1.2 ± 0.2		0.281
CD69+ T cells (%)	93.4 ± 0.9§	84.4 ± 1.4	<0.001	84.0 ± 3.2	0.014	82.3 ± 2.5	0.0002	88.5 ± 1.3	0.028	83.4 ± 4.4	0.017	0.373
CD4+ T cells												
CD4+ T cells/μl	ND			556 ± 90		944 ± 156		564 ± 80		494 ± 97		0.154
IL-2 production (%)	57.6 ± 2.9	35.6 ± 2.1	<0.001	32.1 ± 4.5	0.0002	22.6 ± 2.2	<0.0001	46.7 ± 2.9	0.205	44.6 ± 6.1	0.331	<0.0005
IL-4 production (%)	3.6 ± 0.5	2.2 ± 0.2	0.001	2.2 ± 0.4	0.168	1.2 ± 0.2	<0.0001	3.0 ± 0.5	0.678	2.4 ± 0.5	0.739	0.008
IL-10 production (MFI)	30 ± 3	32 ± 3	0.723	35 ± 6	1.000	14 ± 7	0.707	45 ± 5	0.144	24 ± 8	1.000	0.005
CD25+ CD4 cells (%)	47.8 ± 2.2	57.9 ± 1.9	0.005	58.0 ± 3.5	0.292	56.5 ± 4.2	0.104	61.1 ± 3.3	0.025	55.2 ± 4.9	0.969	0.818
IL-10R+ CD4 cells (MFI)	161 ± 5	171 ± 3	0.158	176 ± 7	0.900	176 ± 7	0.249	166 ± 6	1.000	164 ± 8	1.000	0.391
CD28+ CD4 cells (MFI)	629 ± 5	615 ± 4	0.135	606 ± 14	0.955	629 ± 8	1.000	617 ± 5	0.984	599 ± 8	0.027	0.026
CD154+ CD4 cells (MFI)	67 ± 5	83 ± 4	0.007	85 ± 6	0.117	88 ± 10	0.185	81 ± 5	0.075	76 ± 4	1.000	0.726
CD8+ T cells												
CD8+ T cells/μl	ND			669 ± 97		899 ± 169		404 ± 48		469 ± 84		0.015
IL-2 production (%)	30.0 ± 2.6	19.4 ± 1.4	0.001	15.3 ± 3.2	0.001	17.0 ± 2.2	0.005	26.4 ± 2.5	1.000	17.0 ± 2.7	0.046	0.010
IL-4 production (%)	2.7 ± 0.7	1.6 ± 0.2	0.203	1.9 ± 0.6	1.000	1.4 ± 0.3	0.834	1.9 ± 0.3	1.000	0.9 ± 0.2	0.176	0.127
IL-10 production (MFI)	39 ± 4	34 ± 3	0.205	28 ± 5	0.557	25 ± 5	0.203	51 ± 7	1.000	28 ± 7	1.000	0.021
CD25+ CD8 cells (%)	6.6 ± 1.5	5.3 ± 0.8	0.144	5.6 ± 2.3	1.000	6.9 ± 1.3	1.000	5.5 ± 1.3	1.000	2.2 ± 0.4	0.035	0.071
IL-10R+ CD8 cells (MFI)	141 ± 7	146 ± 4	0.613	143 ± 13	1.000	150 ± 6	1.000	142 ± 5	1.000	149 ± 7	1.000	0.606
CD28+ CD8 cells (MFI)	547 ± 10	501 ± 8	<0.001	488 ± 16	0.004	507 ± 22	0.564	509 ± 7	0.020	495 ± 6	0.002	0.062
CD154+ CD8 cells (MFI)	15 ± 9	4 ± 0	0.060	4 ± 1	1.000	4 ± 1	1.000	4 ± 1	0.744	3 ± 1	0.178	0.711
B cells												
B cells (CD19+)/μl	ND			78 ± 23		78 ± 9		107 ± 19		100 ± 30		0.430
IL-6 production (%)	17.2 ± 2.9	17.4 ± 1.3	0.665	15.7 ± 3.4	1.000	14.0 ± 1.8	1.000	20.2 ± 2.6	1.000	21.0 ± 2.7	0.960	0.116
IL-10 production (MFI)	3 ± 1	5 ± 2	0.842	2 ± 1	1.000	2 ± 1	1.000	5 ± 2	1.000	16 ± 9	0.780	0.102
CD25+ B cells (%)	33.5 ± 2.8	26.5 ± 1.5	0.021	27.7 ± 2.7	1.000	24.5 ± 2.0	0.166	31.1 ± 3.5	1.000	20.7 ± 4.1	0.020	0.161
IL-6R+ B cells (MFI)	6 ± 1	11 ± 2	0.684	25 ± 7	0.057	2 ± 1	0.587	16 ± 4	0.241	1 ± 1	0.145	<0.0001
IL-10R+ B cells (MFI)	76 ± 4	66 ± 5	0.125	76 ± 6	1.000	64 ± 7	0.907	69 ± 11	1.000	49 ± 12	0.372	0.460
CD40+ B cells (MFI)	359 ± 7	339 ± 5	0.063	313 ± 11	0.010	349 ± 10	1.000	333 ± 8	0.268	371 ± 10	1.000	0.008
Monocytes												
Monocytes/μl	ND			592 ± 83		808 ± 157		459 ± 47		407 ± 87		0.053
IL-6 production (%)	44.2 ± 3.4	37.6 ± 1.8	0.028	42.3 ± 3.4	1.000	33.2 ± 3.4	0.053	39.0 ± 3.4	0.875	36.9 ± 4.3	0.770	0.425
IL-10 production (MFI)	22 ± 7	26 ± 3	0.166	25 ± 6	1.000	23 ± 6	1.000	27 ± 6	0.929	36 ± 9	0.381	0.549
IL-6R+ monocytes (MFI)	223 ± 14	194 ± 8	0.027	181 ± 14	0.105	200 ± 14	0.901	203 ± 13	0.692	198 ± 24	0.801	0.747

Table 3. continued

	Controls	Patients	*P** controls versus patients	CsA/Aza	*P*† controls versus CsA/Aza	CsA/MMF	*P*† controls versus CsA/MMF	Tacr/Aza	*P*† controls versus Tacr/Aza	Tacr/MMF	*P*† controls versus Tacr/MMF	*P*‡ patient groups
IL-10R+ monocytes (MFI)	104 ± 10	108 ± 4	0.197	105 ± 18	1.000	107 ± 8	1.000	113 ± 5	0.555	104 ± 12	1.000	0.853
CD40+ monocytes (%)	0.2 ± 0.1	0.3 ± 0.1	0.908	0.2 ± 0.1	1.000	0.4 ± 0.1	1.000	0.4 ± 0.3	1.000	0.5 ± 0.4	1.000	0.827
CD80+ monocytes (%)	0.0 ± 0.0	0.4 ± 0.0	0.308	0.1 ± 0.0	1.000	0.0 ± 0.0	1.000	0.0 ± 0.0	1.000	0.0 ± 0.0	1.000	0.563
CD86+ monocytes (MFI)	443 ± 9	421 ± 6	0.122	403 ± 15	0.156	431 ± 11	1.000	423 ± 9	0.676	433 ± 14	1.000	0.272
NK cells (CD56+)/µl	ND			124 ± 53		198 ± 33¶		69 ± 15		77 ± 15		0.001
CD4 helper activity (%)	ND			92 ± 17		59 ± 13		104 ± 15		59 ± 21		0.073
sCD30 (U/ml)	ND			59 ± 8		46 ± 7		41 ± 4		41 ± 5		0.292
Neopterin (nmol/g S-Cr)	ND			1704 ± 243		1401 ± 309		1121 ± 157		1153 ± 111		0.069

Seventy-eight patients had functioning grafts two years post-transplant. Data of one patient treated with sirolimus are not included in the Table.

CsA, cyclosporine A; Aza, azathioprine; MMF, mycophenolate mofetil; Tacr, tacrolimus; sCD30, serum soluble CD30; ND, not done.

*Wilcoxon rank-sum test was used for statistical comparison of healthy controls and the whole patient group.

†Pairwise comparison holding experiment-wise error, as a *post hoc* test correcting for multiple testing, was used for comparison between controls and each of the four immunosuppressive treatment groups.

‡Kruskall–Wallis H test was used for statistical comparison between the four patient groups.

§Data of double or triple fluorescence analysis are given as mean ± SEM of mean fluorescence intensity (MFI) and % of gated cells, respectively, as indicated.

¶Comparison between each of the patient groups revealed that NK-cell concentrations were enhanced in CsA/MMF patients compared with each other group (CsA/Aza, $P = 0.010$; Tacr/Aza, $P < 0.0005$; Tacr/MMF, $P = 0.010$), whereas no significant differences were detected between the other patient groups. This result coincided with enhanced mononuclear cell counts and also T-cell and monocyte counts in the CsA/MMF patient group compared with the other groups. However, looking at the percentage of T cells, B cells, monocytes and NK cells, respectively, within the mononuclear cell subset, only the percentage of NK cells was enhanced compared with the other patient groups [4.7 ± 1.4%, CsA/Aza; 6.6 ± 0.9%, CsA/MMF; 3.5 ± 0.6, Tacr/Aza; 5.1 ± 0.8%, Tacr/MMF; $P = 0.014$; significant differences between CsA/MMF and CsA/Aza ($P = 0.035$), and also between CsA/MMF and Tacr/Aza ($P = 0.003$)].

Table 4. Immune parameters in renal transplant recipients on CsA- versus Tacr-based immunosuppression and on Aza- versus MMF-based immunosuppression, respectively, analyzed by both univariate (Wilcoxon rank-sum test) and multivariate analyses (stepwise logistic regression).*

Immune parameter	CsA	Tacr	Univariate analysis	Multivariate analysis	Aza	MMF	Univariate analysis	Multivariate analysis
CD4 helper activity (%)	71 ± 11	89 ± 12	0.365	>0.100†	99 ± 11	59 ± 11	0.009	>0.100†
CD4 cell IL-2 (%)	26.6 ± 2.4‡	46.0 ± 2.7	<0.0001	<0.0001	40.8 ± 2.7	30.5 ± 3.1	0.017	>0.100†
CD8 cell IL-2 (%)	16.3 ± 1.8	23.4 ± 2.0	0.007	0.014	21.9 ± 2.1	17.2 ± 1.7	0.187	>0.100†
CD4 cell IL-4 (%)	1.6 ± 0.2	2.8 ± 0.4	0.008	0.046	2.7 ± 0.4	1.6 ± 0.2	0.017	>0.100†
CD4 cell IL-10 (MFI)	27 ± 3	39 ± 4	0.029	>0.100†	41 ± 4	23 ± 3	0.001	0.008
sCD30 (U/ml)	52 ± 5	41 ± 3	0.272	>0.100†	48 ± 4	45 ± 5	0.535	>0.100†
Neopterin (nmol/g S-Cr)	1528 ± 205	1131 ± 113	0.079	>0.100†	1349 ± 140	1318 ± 208	0.807	>0.100†
ISC formation (SAC I) ($ISC/10^6$ B cells)	2556 ± 681	1694 ± 519	0.713	>0.100†	2664 ± 633	1485 ± 548	0.037	0.020
ISC formation (PWM) (%)	46 ± 11	70 ± 13	0.133	>0.100†	77 ± 13	35 ± 10	0.006	0.021
CD28+ CD4 cells (MFI)	619 ± 8	611 ± 4	0.056	>0.100†	613 ± 6	619 ± 6	0.516	>0.100†
CD25+ CD8 cells (%)	6.3 ± 1.2	4.4 ± 0.9	0.151	>0.100†	5.5 ± 1.2	5.4 ± 1.0	0.995	>0.100†
CD40+ B cells (MFI)	334 ± 8	346 ± 7	0.254	>0.100†	325 ± 7	357 ± 8	0.005	>0.100†
IL-6R+ B cells (MFI)	12 ± 3	11 ± 3	0.873	>0.100†	20 ± 4	2 ± 1	<0.0001	<0.0001

ISC, immunoglobulin-secreting cell; CsA, cyclosporine A; Aza, azathioprine; MMF, mycophenolate mofetil; Tacr, tacrolimus.

*Seventy-eight patients had functioning grafts two years post-transplant. Data of one patient treated with sirolimus are not included in the Table. Stepwise logistic regression analysis was performed considering calcineurin inhibitors (Tacr versus CsA), antimetabolites (Aza versus MMF), occurrence of acute rejection, deceased versus living donor, recipient age and 2-year creatinine clearance as independent variables. To minimize problems of multiple testing, multivariate analysis was restricted to parameters previously shown to be predictive of graft outcome (CD4 cell help, CD4 cell IL-4 and IL-10 responses, sCD30, neopterin and ISC formation) and to parameters with meaningful differences in the univariate analysis (CD4 cell IL-2 response, CD8 cell IL-2 response, CD28+ CD4 cells, CD25+ CD8 cells, CD40+ B cells, IL-6R+ B cells).

†CsA versus Tacr and Aza versus MMF, respectively, did not meet the 0.1 significance level for entry into the multivariate model.

‡Data of double and triple fluorescence analysis are given as mean ± SEM of mean fluorescence intensity (MFI) and % of gated cells, respectively, as indicated.

CD4 helper function

Helper activity of CD4+ T cells was highest in Tacr/Aza-treated patients and was generally higher on Aza versus MMF treatment ($P = 0.009$; Tables 3 and 4). However, Aza versus MMF treatment was not confirmed as an independent factor by stepwise logistic regression (Table 4).

Serum sCD30 levels

Although 1-year sCD30 serum levels were previously shown to be downregulated on Tacr compared with CsA treatment [27], the statistical significance of the downregulatory effect of Tacr on sCD30 was lost at 2 years post-transplant (sCD30 ≥ 60 U/ml: 13/38 (34%) with CsA versus 7/40 (18%) with Tacr; $P = 0.091$; Table 3), possibly because Tacr trough levels were somewhat lower at 2 years compared with 1 year post-transplant (8.2 ± 0.4 versus 9.2 ± 0.5 ng/ml, $P = 0.084$) and concomitantly administered antimetabolites (Aza, $P = 0.013$; MMF, $P = 0.003$) or steroids ($P < 0.001$) were significantly lower at 2 years.

Intracellular cytokine production

Compared with CsA, Tacr-based immunosuppression was associated with significantly increased IL-2 responses of

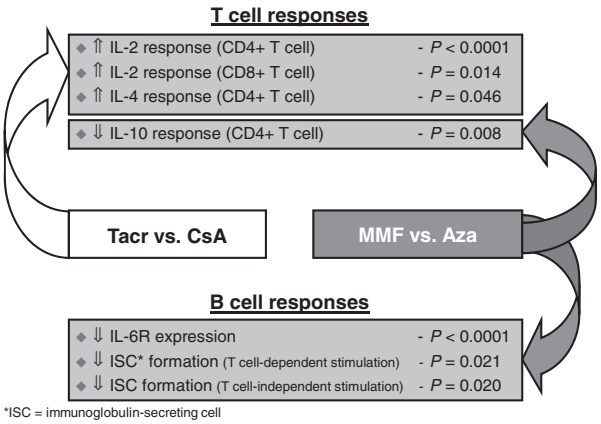

Figure 1 Key results of the impact of tacrolimus (Tacr)- versus cyclosporine A (CsA)-based and mycophenolate mofetil (MMF)- versus azathioprine (Aza)-based immunosuppressive regimens on T- and B-cell responses at 2 years after renal transplantation. The impact of immunosuppressive maintenance regimens at 2 years post-transplant was mainly on T- and B-cell responses, whereas no significant effects were detected on monocyte responses. Tacr-compared with CsA-based regimens provided previously shown graft protective effects on CD4 cell IL-4 responses and were associated with increased T-cell IL-2 production, whereas MMF- compared with Aza-based regimens significantly suppressed B cells responses and CD4 cell production of the B-cell factor IL-10. *P* values were calculated using stepwise logistic regression.

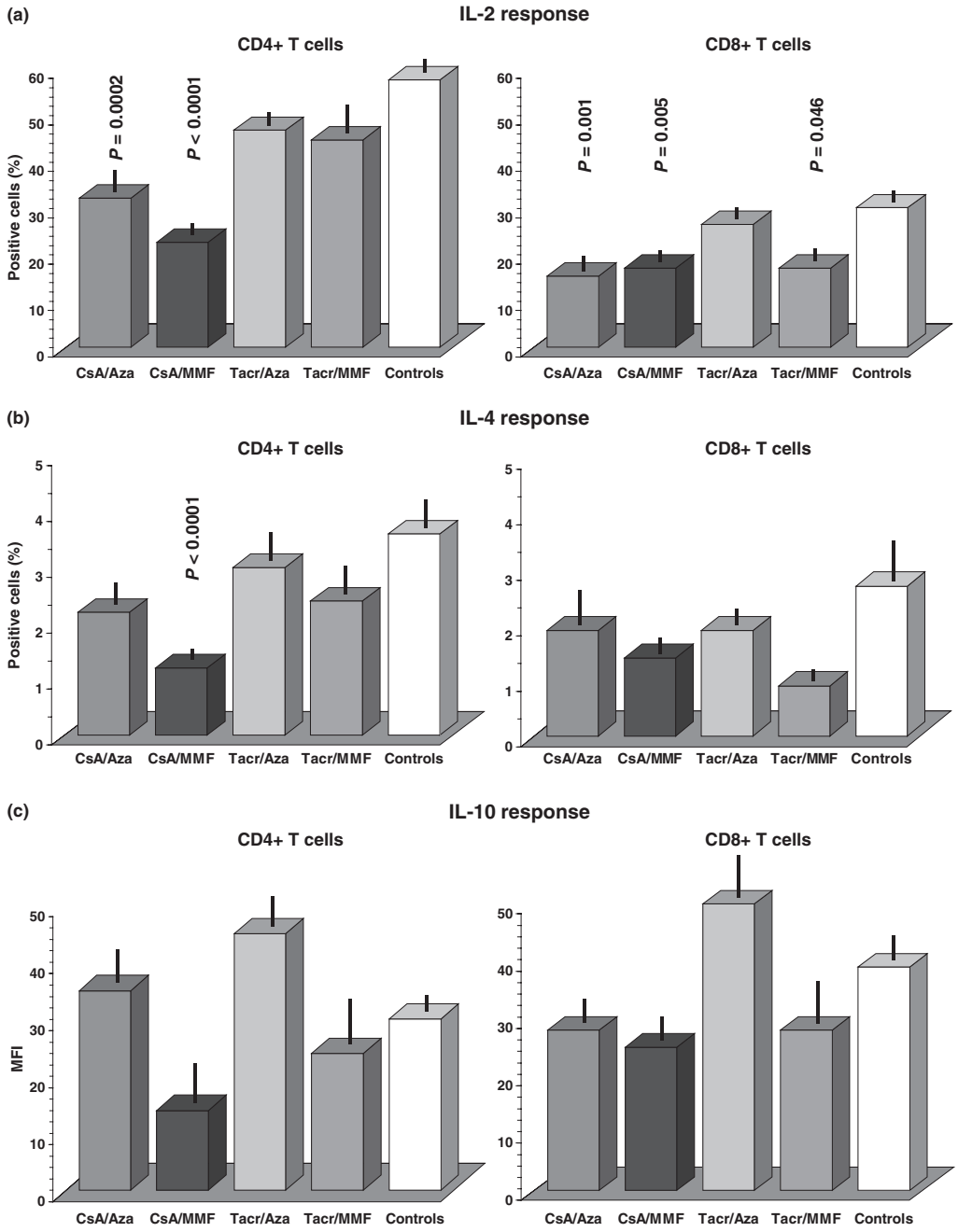

Figure 2 Intracellular IL-2 (a), IL-4 (b) and IL-10 (c) production of CD4+ T cells and CD8+ T cells in healthy controls as compared with the four patient groups treated with cyclosporine A/azathioprine (CsA/Aza), cyclosporine A/mycophenolate mofetil (CsA/MMF), tacrolimus/azathioprine (Tacr/Aza) and Tacr/MMF, respectively, at 2 years post-transplant.

Compared with healthy controls, only Tacr/Aza-treated patients showed no significantly decreased T-cell IL-2 or IL-4 responses. Tacr-based immunosuppression was associated with increased IL-2 responses (CD4+ T cells, $P < 0.0001$; CD8+ T cells, $P = 0.007$) compared with CsA treatment. Interestingly, Tacr/Aza-treated patients showed a significantly enhanced IL-2 production of CD8+ T cells compared with all other treatment groups (CsA/Aza, $P = 0.004$; CsA/MMF, $P = 0.013$; Tacr/MMF, $P = 0.033$). CD4 cell IL-4 responses were higher in Tacr than in CsA-treated patients ($P = 0.008$), whereas MMF therapy was associated with reduced CD4 cell IL-4 ($P = 0.017$) and IL-10 production ($P = 0.001$) compared with Aza. The highest T-cell responses of the B-cell factor IL-10 were observed on Tacr/Aza therapy as compared with the other treatment groups (CD4 cells: $P = 0.001$, CsA/MMF; $P = 0.032$, Tacr/MMF; CD8 cells: $P = 0.021$, CsA/Aza; $P = 0.006$, CsA/MMF; $P = 0.044$, Tacr/MMF). Mean and SEM are indicated. *P* values are given for statistically significant differences between healthy controls and patient groups (pairwise comparison holding experiment-wise error as a *post hoc* test correcting for multiple testing).

both CD4+ and CD8+ T cells (lreg: $P < 0.0001$, CD4 cells; $P = 0.014$, CD8 cells; Table 4, Fig. 2a). As a result, both CD4 and CD8 cell IL-2 production in Tacr/Aza-treated patients reached the level of healthy controls, whereas CsA-treated patients showed profoundly lower IL-2 production than controls (Figs 1 and 2a, Table 3). With respect to T-cell IL-4 responses, Tacr compared with CsA treatment was confirmed by multivariate analysis to be independently associated with enhanced CD4-cell IL-4 production ($P = 0.046$, lreg; Table 4, Figs 1 and 2b). Patients on MMF showed reduced CD4 cell IL-10 responses compared with Aza (lreg: $P = 0.008$; Table 4, Figs 1 and 2c). The highest CD4 and CD8 cell responses of the B-cell factor IL-10 were observed in patients on Tacr/Aza.

Compared with healthy controls, the patients' T-cell proliferative capacity, as assessed by CD69 expression upon stimulation with PMA and ionomycin, was significantly downregulated in all treatment groups (Table 3) without significant differences between the groups. This suppressed T-cell proliferative capacity, however, coincided with a profound suppression of CD4 and CD8 cell IL-2 production only in CsA-treated patients (Table 3). CD4 cell IL-4 production was strongly suppressed only in CsA/MMF-treated patients (compared with controls $P < 0.0001$; Table 3).

Impact of immunosuppression on B cells

Flow cytometry

Upregulated CD40 expression on B cells in MMF compared with Aza-treated patients (univariate $P = 0.005$) was not confirmed in the multivariate analysis (Table 4). The profoundly suppressed B-cell IL-6R expression in MMF compared with Aza-treated patients (univariate $P < 0.0001$), however, was proven to be an independent effect of MMF treatment ($P < 0.0001$, lreg; Table 4 and Fig. 1).

ISC formation

B-cell responses were significantly lower in MMF compared with Aza-treated patients and MMF was proven to be an independent factor in multivariate analysis, both after T-independent stimulation of B-cell cultures with SAC I ($P = 0.020$) and T-dependent stimulation of allogeneic cocultures with PWM ($P = 0.021$; Table 4, Figs 1 and 3).

Intracellular cytokine production

B-cell IL-6 and IL-10 responses were enhanced in Tacr/MMF patients (IL-6: $P = 0.025$ versus CsA/MMF; IL-10: $P = 0.033$ versus CsA/Aza, $P = 0.028$ versus CsA/MMF), which may be attributed to an increased rate of acute rejection in this patient group. Compared with healthy controls, the patients' B-cell IL-6 and IL-10 production were not significantly different (Table 3).

Antibody formation against HLA class I and II antigens

As we found HLA antibodies in only 14 patients pretransplant and in eight patients at 2 years post-transplant, statistically significant associations with maintenance immunosuppressive regimens could not be found. However, three of seven patients who lost their pretransplant detectable HLA antibodies were on Tacr/MMF (CsA/Aza, $n = 2$; CsA/MMF, n = 1; Tacr/Aza, $n = 1$; Tacr/MMF,

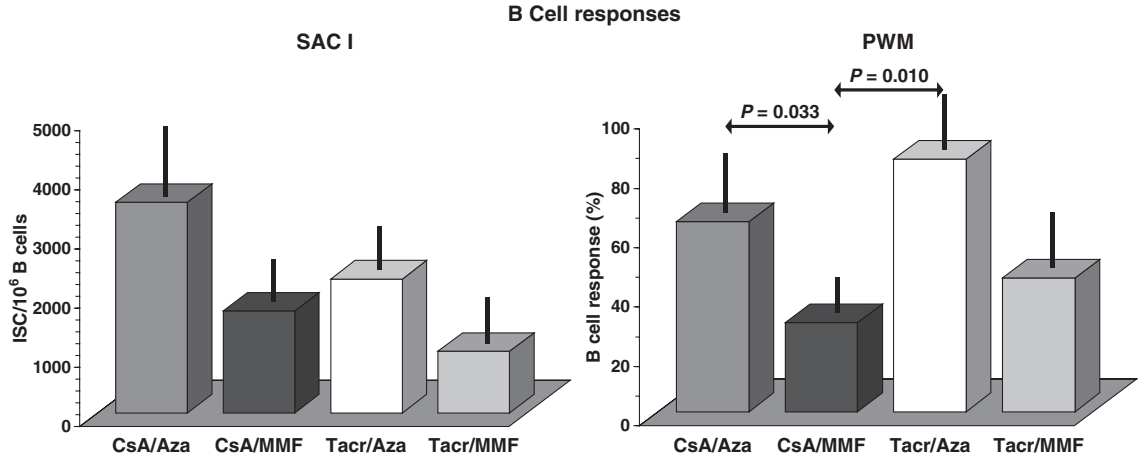

Figure 3 *In vitro* B-cell responses in the four patient groups treated with cyclosporine A/azathioprine (CsA/Aza), cyclosporine A/mycophenolate mofetil (CsA/MMF), tacrolimus/azathioprine (Tacr/Aza) and Tacr/MMF, respectively, at 2 years post-transplant.
Immunoglobulin-secreting cell (ISC) formation was significantly lower in MMF compared with Aza-treated patients, both after T-independent stimulation of B-cell cultures with SAC I ($P = 0.037$) and T-dependent stimulation of allogeneic T- and B-cell cocultures with PWM ($P = 0.006$). Mean and SEM are indicated. *P* values are given for statistically significant differences between patient groups (Wilcoxon rank-sum test).

$n = 3$), but none of the seven patients with persisting HLA antibody formation (CsA/Aza, $n = 1$; CsA/MMF, $n = 3$; Tacr/Aza, $n = 2$; Sirolimus, $n = 1$) thereby suggesting a favorable impact on HLA antibody formation by Tacr/MMF treatment. Only one patient developed de-novo HLA antibodies (CsA/MMF).

Impact of immunosuppression on monocytes

Flow cytometry

Compared with healthy controls, IL-6R was slightly downregulated on the patients' monocytes ($P = 0.027$; Table 3). Significant differences of cytokine receptor or costimulatory ligand expression between the patient groups were not detected (Table 3).

Serum neopterin levels

Tacrolimus versus CsA treatment was not found to be associated with neopterin downregulation in multivariate analysis (Tables 3 and 4).

Intracellular cytokine production

Monocyte IL-6 and IL-10 responses were not significantly different between the patient groups. Monocyte IL-6 production was somewhat lower in CsA/MMF-treated patients as compared with healthy controls ($P = 0.053$; Table 3).

Discussion

To our knowledge, this is the first prospective study of the impact of immunosuppressive maintenance regimens on Th2, B-cell and monocyte responses, which have been shown previously to predict graft outcome [9–27]. Maintenance immunosuppression with Tacr/MMF showed the most potent graft protective effects on these parameters in multivariate analysis (enhanced CD4 cell IL-4 production by Tacr; decreased CD4 cell IL-10 production, B-cell IL-6R expression and ISC formation by MMF) which may provide an explanation for the better clinical results of this regimen in the Symphony study. Based on our findings, it has to be considered, however, that in patients with a low immunological risk profile, a Tacr/MMF regimen may induce overimmunosuppression. Instead, a CsA/Aza or Tacr/Aza regimen may exert sufficient immunosuppression, thereby allowing for a more physiological state of the patient's immune response. It is well known that overimmunosuppression increases the risk of side effects such as malignancy, BK nephropathy and other infectious diseases which may – through activation of Toll-like receptors [30] or the necessary dose reductions of immunosuppressive drugs [31,32] – negatively affect graft survival in the long-term. Consistent with this hypothesis are the 3-year data of the Symphony study, which do no longer demonstrate a superiority of Tacr/MMF [33], and data of the Collaborative Transplant Study [34] showing comparable 5-year graft survival between CsA and Tacr and also between mycophenolic acid and Aza regimens.

Taking these considerations into account, what are the clinical implications of our results? We conclude that we have shown differential effects of four immunosuppressive treatment regimens on clinically relevant and predictive immune parameters. These data should contribute to allow personalized immunosuppressive treatment based on the *in vitro* assessment of these parameters with the potential to improve graft and patient outcome in the long term. Parameters of donor-specific immune reactivity, especially *de novo* HLA antibody formation, could be advantageously added for monitoring. For validation of this concept, a large randomized study with long-term follow-up will be necessary, comparing personalized immunosuppressive treatment as outlined herein with the low-dose Tacr and MMF regimen of the Symphony study, which is currently considered the most effective treatment regimen.

We recognize that the study has limitations. For reasons of the patients who had to be switched to another immunosuppressive regimen out of clinical necessity, the clear and randomized prospective design of this pilot study could unfortunately not be maintained throughout. However, the results are reasonably robust because the influence of possible confounders – as identified by detail-analysis of the 2-year treatment groups – was accounted for by multivariate testing. Moreover, the 2-year post-transplant examination point largely rules out interfering events resulting from acute rejection, CMV activation or switches of immunosuppressive regimens which nearly all occurred during the first 18 months. To further minimize the likelihood of statistical error, multivariate analysis of immunosuppressive treatment effects was restricted to parameters previously shown to be predictive of graft outcome and to parameters with clinically meaningful differences by univariate analysis (altogether 13 parameters, Table 4). Additionally, a *post hoc* test correcting for multiple testing was used for comparison of controls with each of the treatment groups.

With respect to technical quality and reliability of intracellular cytokine determination, results were hugely satisfying as shown in Table 2. However, the precise setting of gates, exclusion of CD19 and CD14 double positive cells, and preincubation with rat IgG Fc fragments appear necessary especially for valid and reliable monokine determination.

Our data show that patients on CsA/Aza or Tacr/Aza, as compared with MMF regimens, possess a stronger

humoral immune response (CD4 cell IL-10 production, B-cell IL-6R expression as well as ISC formation), which in turn may reduce the risk of infectious disease complications. Furthermore, we observed physiological T-cell IL-2 and IL-4 responses on Tacr/Aza, but not on CsA-based treatment. Tacr/Aza might therefore be the treatment of choice in patients at risk of infectious disease such as elderly patients. On the other hand, the response of the B-cell differentiation factor IL-10 was highest in Tacr/Aza patients – which was already described at 4 months post-transplant in this prospective study [11] – with a tendency to even exceed the CD4 cell IL-10 response of healthy controls. This finding coincided with early graft losses because of acute humoral rejection in three Tacr/Aza patients. It follows that Tacr/Aza cannot be recommended in patients at risk of acute humoral rejection. Instead, a combination of Tacr with MMF may be preferred for downregulation of humoral responses including IL-10. As an alternative, a mTOR inhibitor might be combined with Tacr, as Niemczyk *et al.* [35] described decreased T-cell IL-10 expression on sirolimus in a small group of liver and kidney transplant recipients.

In Tacr compared with CsA-treated patients, we found significantly increased CD4 and CD8 cell IL-2 and CD4 cell IL-4 responses. Indeed, Tacr/Aza patients showed physiological responses as compared with healthy controls, whereas a profound downregulation of IL-2 responses was found in CsA-treated patients, and of CD4 cell IL-4 responses in CsA/MMF patients. CD25 (IL-2R alpha) expression on CD4 cells, on the other hand, was enhanced in the whole patient group, probably an indication of counterregulation. Previous data of our group showed that IL-2 responses were not predictive of graft outcome [9–11] and hence, a negative impact may not be expected.

In contrast to our findings at 2 years post-transplant, Rostaing *et al.* [36] found significantly lower intracellular T-cell IL-2 production in a small group of seven Tacr as compared with seven CsA-treated patients up to 6 months post-transplant. In a previous study of 20 stable renal transplant recipients who were switched from CsA to Tacr for non-immunologic reasons, we found a decrease in phytohaemagglutinin (PHA), but not in PWM-stimulated T-cell IL-2 responses together with decreased CD4 helper activity, but an increase in CD4 cell IL-10 and LPS-stimulated monokine responses, using ELISA techniques for cytokine assessment in culture supernatants [28]. The more pronounced effect of Tacr treatment on the suppression of IL-2 responses and CD4 help may be explained by the increased Tacr trough levels in the previous study as compared with the current study, whereas the reported increase in CD4 cell IL-10 responses in mainly Tacr/Aza-treated patients [28] is consistent with our current data.

Increased T-cell IL-4 production in Tacr compared with CsA-treated patients has been previously described by Zamauskaite *et al.* [37] in a group of 26 renal transplant recipients with stable function and we were able to confirm these results for CD4+ T-cell IL-4 production. With respect to the predictive value of pretransplant CD4 cell IL-4 responses, we found previously enhanced pretransplant responses to be associated with a low risk of acute rejection [11] suggesting a favorable effect of Tacr compared with CsA treatment. IL-4 provides an essential cytokine for B-cell activation and induction of the Th2 response, but has also anti-inflammatory properties and may inhibit initial B-cell activation [38]. Tacr upregulates STAT6 in the presence of IL-4, which is thought to play a role in Tacr-mediated protection against rejection [39]. Further support of a graft protective effect by IL-4 is provided by data of Hackstein *et al.* [40] who found decreased kidney graft survival in patients expressing the IL-4R alpha-chain variant which is hyporesponsive to IL-4. However, these data were not confirmed by the Collaborative Transplant Study [41].

Previously, analysis of pretransplant, 4-month and 1-year post-transplant immune parameters within this prospective study has shown that sCD30 and neopterin at 1 year post-transplant predict graft deterioration as a result of chronic graft dysfunction (IF/TA). Tacr compared with CsA treatment effectively suppressed these responses and might therefore be of advantage in patients with elevated sCD30 or neopterin [27,42]. At the 2-year post-transplant time point, however, there was only a non-significant tendency left of lower sCD30 levels on Tacr treatment, and the significantly lower neopterin levels were not shown to be independently associated with Tacr treatment by multivariate analysis. Besides the tendency to lower Tacr levels at 2 years, the significantly lower dosage of immunosuppressive comedication (Aza, MMF, steroids) administered at 2 years compared with 1 year post-transplant might provide an explanation.

It is a main goal in kidney transplantation to prevent chronic allograft dysfunction and thereby prolong graft and patient survival. An individualized immunosuppressive regimen may provide a tool to realize this goal. For immunological risk estimation and selection of an appropriate recipient-tailored immunosuppressive regimen, however, it may not be sufficient to consider the pretransplant immunization status of the recipient and the degree of HLA matching between donor and recipient. Enhanced monocyte activation, CD4 helper activity, sCD30 levels, CD4 cell responses of the B-cell growth and differentiation factor IL-10, low CD4 cell IL-4 responses and enhanced *in vitro* ISC formation provide parameters of increased humoral immune reactivity, which have been

shown to predict a worse graft outcome [9–11,14–18,27]. It is not surprising that these immune parameters of the humoral pathway were found to be predictors of graft outcome, as growing evidence is forthcoming to show that the post-transplant formation of donor-specific antibodies may play a major role in the development of chronic graft dysfunction [19–23].

For the purpose of selecting an appropriate recipient-tailored immunosuppressive regimen based on immunological testing, it appears necessary to know the recipient's underlying immune reactivity and to select an immunosuppressive regimen, which targets those immune functions that are associated with an increased risk of immunological allograft damage. In this study, we were able to show the impact of different maintenance regimens on clinically relevant immune parameters in an attempt to provide an immunological basis for individualized immunosuppression.

Authorship

RW: participated in research design, performance of the research, data analysis and writing of the paper; contributed new reagents or analytic tools. SD: participated in performance of the research and data analysis. HD: participated in performance of the research and data analysis; contributed new reagents or analytical tools. FR, VD, SK-E, WE and WP: participated in performance of the research. R-HB: participated in statistical analysis of data. GO: participated in research design, data analysis and writing of the paper.

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