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ORIGINAL ARTICLE

ATG induction therapy: long-term effects on Th1 but not on Th2 responses

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Summary

Antithymocyte globulin (ATG) induction therapy is associated with an increased long-term risk of infection- and cancer-related death. To analyze long-term effects of ATG induction on lymphocyte function, we prospectively assessed CD4 helper function, B-cell/monocyte and cytokine responses in 84 renal transplant recipients (ATG, $n = 44$) up to 1 year post-transplant. A PWM-driven allogeneic coculture system was used to assess helper function of CD4+ T cells and T-cell-dependent B-cell responses. SAC I was used for T-cell-independent stimulation of B-cell cultures. *In vitro* cytokine secretion and serum soluble CD30 (sCD30) were determined by enzyme-linked immunosorbent assay (ELISA). ATG induced a persistent decrease of peripheral blood lymphocyte counts compared with non-ATG treatment because of a predominant decrease of CD4+ T cells (4 months, 1 year; $P < 0.0005$) which was associated with a decreased CD28 expression (1 year, $P = 0.02$) and CD4 cell interleukin 2 (IL-2) response (4 months, $P < 0.0005$). However, Th2 responses (CD4 help, CD4 cell IL-4 and IL-10 responses, sCD30), which proved to be predictive of graft outcome, were not affected, and neither was the secretion of the lymphoma growth factors IL-6 and IL-10 by B cells and monocytes. Our data show that ATG induction therapy in immunological high-risk patients induces a profound long-term decrease in cell counts and Th1 but not Th2 responses of CD4+ T cells which may explain long-term effects on infection and post-transplant lymphoproliferative disease (PTLD) incidence because of inadequate T-cell control.

Introduction

Antithymocyte globulins (ATG) are widely used as induction therapy in immunological high risk patients in order to prevent acute rejection episodes [1–3]. Apart from short-term effects, ATG induces a prolonged decline of the CD4/CD8 ratio up to 5 years after treatment [4], a consequence of CD4+ T-cell depletion and an increased regeneration of CD8+ T cells. The question arises whether ATG induces not only a depletion of CD4+ T cells but also long-term changes in CD4 cell function, which might be relevant with respect to acute or even chronic rejection, the incidence of infections, or post-transplant lymphoproliferative disease (PTLD). The risk of infectious complications and PTLD was shown to be significantly elevated in patients who received rejection prophylaxis with anti-lymphocyte antibodies [5–7]. The increased PTLD risk might be a long-term effect of ATG on IL-6 or IL-10 secretion. Both cytokines appear to play a role as growth factors in Ebstein Barr virus (EBV)-associated PTLD [8–12].

Soluble CD30 (sCD30) provides a marker for the activation state of Th2 cytokine producing T cells and was shown to be predictive of graft outcome in an analysis of 3899 cadaver kidney recipients within the Collaborative Transplant Study [13]. High pretransplant sCD30 was associated with a significantly decreased 5-year graft survival rate. These data are in accordance with our previous results regarding the Th2 cytokine IL-10. In a prospective study of renal transplant patients, we found that low pretransplant CD4 helper activity and low *in vitro* IL-10 responses predict a low incidence of acute graft rejection and improved 3-year graft function [14]. Immunological graft failure because of acute or chronic rejection occurred only in patients with pretransplant normal CD4 helper activity and elevated IL-10 responses. In line with these data is the report of Merville *et al.* [15] who found a preferential production of IL-10 by graft-infiltrating CD4 cells in renal allograft recipients with accelerated vascular rejection, and data in animal models showing no prolongation of cardiac or pancreatic allograft survival or even acceleration of rejection after IL-10 treatment [16–18]. In the current prospective series of transplants, we previously studied immunological effects of three different immunosuppressive protocols (CsA/Aza, CsA/MMF, Tacr/Aza). The analysis of 4-month data showed that pretransplant CD4 helper defects, low IL-10 responses and enhanced IL-4 responses of CD4 cells predicted a low risk of acute rejection [19], confirming the results obtained in our first study [14].

In the present analysis, we investigated the effects of ATG induction therapy in immunological high-risk patients 4 months and 1 year post-transplant. Besides mononuclear cell subsets and serum sCD30, T and B-cell functions such as CD4 helper activity, *in vitro* immunoglobulin-secreting cell (ISC) formation, and cytokine responses were examined.

Materials and methods

Patients

Eighty-four renal allograft recipients cadaveric transplants (CAD), $n = 63$; living transplants (LIV), $n = 21$), who were transplanted in the Giessen transplant center, were prospectively randomized to one of three immunosuppressive regimens (Table 1). Forty-four of the 84 patients received prophylactic rabbit ATG (Fresenius, Oberursel, Germany; 4 mg/kg/day) induction therapy postoperatively because of an increased immunological risk profile (acute renal failure, PRA >5%, or retransplantation; $n = 37$) or simultaneous islet/kidney transplantation ($n = 8$; Table 1). The mean duration of ATG treatment was 8.6 ± 0.4 days. Twelve of the 22 (55%) acute rejection episodes were confirmed by renal biopsy. Immunological tests were performed pretransplant, 4 months and 1 year post-transplant. The patient groups were comparable with respect to basic immunosuppressive therapy, donor and recipient age, human leukocyte antigen (HLA) matching (Table 1), renal diseases (including insulin-dependent diabetes mellitus) and pretransplant cytomegalovirus (CMV) serostatus of donors and recipients. CMV prophylaxis was performed in CMV-IgG negative recipients of CMV-IgG positive organs [ATG: 10/44 (23%) patients; non-ATG: 8/40 (20%) patients; NS] by intravenous administration of a CMV hyperimmunoglobulin preparation [Cytoglobin 5% (Bayer, Leverkusen, Germany), 4 ml/kg] pretransplant and six times post-transplant in weekly intervals. All other patients received IgG prophylaxis instead.

Table 1. Characteristics of patients who received ATG induction therapy (ATG group) or no ATG (non-ATG group).

	ATG	Non-ATG	*P*-value*
Number of patients	44	40	
Recipient age (years)	48 ± 2	44 ± 2	NS
RRT (% HD/PD/PR)	86/9/5	85/13/3	NS
Waiting time (months)	56 ± 7	63 ± 15	NS
LIV (%)	14% (6)	38% (15)	0.01
SIK (%)	18% (8)	0% (0)	<0.005
Number of retransplants	27% (12)	0% (0)	0.002
PRA	11 ± 2%	4 ± 1%	<0.01
Blood transfusions	6.3 ± 2.0	1.8 ± 0.6	<0.01
Pregnancies	36% (16)	18% (7)	0.05
Donor age (years)	47 ± 2	47 ± 2	NS
HLA-A,B,DR mismatches	3.0 ± 0.2	2.0 ± 0.2	NS
HLA-B,DR mismatches	2.1 ± 0.2	1.4 ± 0.2	NS
Cold ischemia time (hours)	15.2 ± 1.0	8.9 ± 1.0	<0.001
Immunosuppressive therapy			
CsA/Aza/P	34% (15)	25% (10)	NS
CsA/MMF/P	34% (15)	40% (16)	NS
Tacr/Aza/P	32% (14)	35% (14)	NS

*Wilcoxon rank sum test was used for statistical comparison of ATG and non-ATG patients.

RRT, renal replacement therapy [percentage of patients treated with hemodialysis (HD), peritoneal dialysis (PD) and preemptive transplantation (PR)].

LIV, percentage of patients receiving living-related or unrelated transplants ($n = 21$); SIK, simultaneous islet/kidney transplants ($n = 8$).

PRA, peak panel reactive antibodies pretransplant; PRA >5% were found in 18/44 (41%) ATG-treated patients compared with 5/40 (13%) non-ATG treated patients ($P < 0.005$).

Initial immunosuppressive therapy: Aza, azathioprine; P, prednisolone; Tacr, tacrolimus; Immunosuppressive therapy 4 and 12 months post-transplant did not differ significantly between the ATG and non-ATG groups (data not shown).

Flow cytometric analysis

Mononuclear cell subsets were determined by double-fluorescence laser flow cytometry as described previously

[20,21]. The following monoclonal antibodies were used: OKT4-FITC (CD4), OKT8-FITC (CD8) and OKT11-FITC (CD2; Ortho, Raritan, NJ, USA); anti-ICAM-1 (CD54) and anti-LFA-1 (CD11a/CD18; R&D Systems, Minneapolis, MN, USA); anti-CD28-PE, anti-CD25, anti-CD56, anti-LFA-3 (CD58) and anti-B7-1-PE (CD80; Becton Dickinson, Sunnyvale, CA, USA); anti-TNF-αR (Alexis Biochemicals, Lausanne, Switzerland); anti-IFN-γR (Serotec, Düsseldorf, Germany); OKT3-FITC (CD3), OKT3-PE, Leu12-FITC (CD19), LeuM7-FITC (CD13) and LeuM7-PE were used for the staining of T cells, B cells and monocytes respectively. Incubation with antibodies was performed at 4 °C and phosphate-buffered saline containing 0.1% NaN_3 was used as washing solution. All measurements were performed using a FACSCalibur flow cytometer (Becton Dickinson). An Ortho Cytoron flow cytometer was used to assess absolute peripheral blood mononuclear cells (PBMC) counts.

Serum sCD30

Sera were thawed and tested for sCD30 content by ELISA (Bender MedSystems, Vienna, Austria) as described previously [13].

Cell cultures

Cell separations were performed as described previously [22–24]. Briefly, PBMC were separated into 'whole' T and non-T cells (termed B cells in the following) by the rosetting technique. Monocytes were not especially depleted. Whole T cells were depleted from CD8 cells (CD4+ T-cell subset) by treatment with OKT8 monoclonal antibody and rabbit complement. T-cell enriched subsets (called T cells or 'whole' T cells) were 92–96% CD2+, <2% CD19+ and <1% CD13%. CD4 cell enriched subsets (called CD4+ T cells or CD4 cells) were 73–84% CD4+ and ≤5% CD8+.

The pokeweed mitogen (PWM)-stimulated allogeneic cocultures of control B cells with patient T cells or the CD4+ T-cell subset, PWM-stimulated cocultures of patient B and control T cells (T-cell dependent stimulation of B cells by PWM), and staphylococcus cowan strain I (SAC I)-stimulated B-cell cultures (T-cell and monocyte independent stimulation of B cells by SAC I) were performed as described previously [22–24]. B-cell differentiation was assessed in a reverse hemolytic plaque assay. B cells (10^5) and T cells (10^5) alone were cultured with PWM in order to control the quality of cell separation. ISC responses did not exceed those of unstimulated control T or B-cell cultures, indicating a functionally sufficient separation [22]. T helper activity and B-cell functions were calculated from the results (ISC/10^6 B cells) of the following cocultures:

T cell or CD4 cell helper activity:

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

PWM-stimulated B-cell function:

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

SACI-stimulated B-cell function *:*

$$[B(P)+SACI]-[B(P)+M]$$

where B(C),T(C) is the B and T cells of a control; B(P),T(P), patient B or T cells (or CD4+ T cells); M, culture medium. Values of <10% helper function were defined as defective helper activity.

Cytokine secretion

Frozen supernatants of PWM-stimulated allogeneic T and B-cell cocultures and SAC I-stimulated B-cell cultures were thawed and tested for cytokine content. Double sandwich ELISA were used for testing of IL-2 (human IL-2 Quantikine; standards 31.3–2000 pg/ml), IL-4 (human IL-4 HS Quantikine; standards 0.25–16 pg/ml), IL-10 (human IL-10 Quantikine; standards 7.8–500 pg/ml; R&D Systems, Minneapolis, MN, USA) and IL-6 (IL-6 Milenia; standards 15.6–1000 pg/ml; Biermann, Bad Nauheim, Germany).

Cytokine responses of PWM-stimulated allogeneic cocultures and SAC I-stimulated B-cell cultures were calculated by subtracting the cytokine responses of the respective unstimulated cultures. Monocyte and B-cell cytokine responses (IL-6, IL-10) were measured in supernatants of unstimulated B-cell/monocyte cultures as well as in unstimulated and PWM-stimulated cocultures of control T with patient 'B cells' (including monocytes). Supernatants of SAC I-stimulated B-cell cultures were tested for B-cell cytokine content (IL-6, IL-10). T-cell cytokine secretion (IL-2, IL-4, IL-10) was assessed in the supernatants of unstimulated and PWM-stimulated cocultures of control B with patient T cells.

T cells produced only between 1% and 19% of B-cell/monocyte derived IL-10 in the PWM-stimulated coculture system. Control B cells/monocytes secreted between 2040 and 2500 pg/ml IL-10 if normal T helper activity was provided. Therefore, a low IL-10 response in a coculture of patient T with control B cells reflects reduced T-cell help for B-cell/monocyte IL-10 secretion.

Statistics

Data are expressed as mean ± SEM. Wilcoxon rank sum test, Wilcoxon signed rank test, chi-square test, Fisher's

exact and Pearson's correlation coefficient were used for statistical analysis. $P \leq 0.05$ were considered statistically significant.

Results

Transplant data and 1-year outcome

As ATG induction therapy was performed in patients with an increased immunological risk profile, immunological risk parameters were more frequent in the ATG compared with the non-ATG group (Table 1). The prolonged cold ischemia time in ATG-treated patients was associated with an increased frequency of acute post-transplant renal failure (defined as need to perform at least one hemodialysis procedure in the first post-transplant week; ATG: 27% (12/44) versus non-ATG: 10% (4/40); $P < 0.05$]. Statistical analysis, however, revealed no association between cold ischemia time and the immune parameters tested. HLA matching was comparable in the two patient groups (Table 1).

One-year patient survival (100%) and 1-year graft survival (ATG: 93%; non-ATG: 98%) were not significantly different between the ATG and non-ATG groups. Three grafts were lost because of acute vascular rejection (ATG group) and one because of venous thrombosis of the graft (non-ATG group). We found no significant difference in the 1-year acute rejection incidence between ATG-treated immunological risk patients and non-ATG patients (ATG: 25%; non-ATG: 28%). However, acute vascular rejection [ATG: 9% (4/44); non-ATG: 0% (0/40)] and late acute rejections [5–12 months post-transplant; ATG: 9% (4/44); non-ATG: 3% (1/40)] occurred more frequently in the ATG group, but the difference did not reach statistical significance.

As living-related or unrelated transplants were more frequent in the non-ATG group (Table 1), it is not surprising that we found a significantly higher peak creatinine clearance during the first 3 months post-transplant in non-ATG patients (ATG: 56 ± 4 ml/min versus non-ATG: 76 ± 6 ml/min; $P = 0.01$). Four months and 1 year after transplantation there was no significant difference in graft function between the ATG and non-ATG groups (1-year creatinine clearance: 44 ± 3 ml/min, ATG; 52 ± 6 ml/min, non-ATG). We found no significant difference in the anti-CMV-IgG serostatus of donors and recipients pretransplant (data not given), and the incidence of symptomatic CMV infections in the first post-transplant year was not increased in the ATG group [ATG: 32% (14/44); non-ATG: 43% (17/40); NS].

Four-month and 1-year data showed no significant differences with respect to systolic or diastolic blood pressure, number of antihypertensive drugs, incidence of proteinuria, serum cholesterol and triglyceride levels, and administration of lipid lowering agents (data not shown).

Mononuclear cell subsets

In ATG-treated patients, PBMC counts were significantly lower 4 months and 1 year post-transplant when compared with non-ATG patients ($P < 0.0005$; Table 2). These low PBMC counts in ATG-treated patients were because of a strong absolute decrease of CD4+ T-cell counts 4 and 12 months post-transplant compared with the pretransplant values ($P = 0.002$, Table 2) whereas absolute CD4+ T-cell counts did not decrease in non-ATG patients. As the CD8+ T-cell counts in both patient groups did not significantly change 4 and 12 months post-transplant compared with the pretransplant values, the CD4/CD8 ratio showed a profound decrease in ATG-treated patients up to 1 year post-transplant ($P < 0.0005$; Table 2). The CD4/CD8 decrease in non-ATG patients was not as strong as in ATG patients as given by the highly significant differences between ATG and non-ATG patients (Table 2). The relative increase in the percentage of monocytes in ATG patients ($P = 0.01$; Table 2) was not because of a significant increase of absolute monocyte counts (Table 2).

Expression of co-stimulatory ligands, adhesion molecules and cytokine receptors

Whereas CD80 expression on monocytes was not significantly diminished in ATG-treated patients, the CD28 expression on T cells was reduced up to 1 year post-transplant ($P < 0.02$; Table 3).

Symptomatic CMV infections in ATG or non-ATG patients did not result in an increased expression of adhesion molecules 1 year post-transplant. In ATG-treated patients, absolute CD54+ and also CD58+ monocyte counts increased 4 and 12 months post-transplant compared with the pretransplant cell counts ($P = 0.001$ and 0.02, respectively; Table 3). The increase in CD54+ monocyte counts of non-ATG patients just reached significance ($P = 0.05$, Table 3) and the post-transplant percentage of CD54+ monocytes was significantly diminished in non-ATG compared with ATG patients ($P < 0.05$; Table 3). The post-transplant percentage of CD54+ and also of CD11a/CD18+ T cells in ATG-treated patients showed an increase compared with non-ATG patients, whereas absolute cell counts were not significantly different or even decreased (CD11a/CD18+ T cells) because of the decreased absolute T-cell counts in ATG compared with non-ATG patients (Tables 2 and 3). The increased expression of adhesion molecules – as late as 4 months and even 1 year post-transplant – in the immunological high-risk ATG

Table 2. Mononuclear cell subsets pretransplant, 4 months, and 1 year post-transplant in patients with or without ATG induction therapy.

	Pretransplant	4 months	1 year	*P*-value*
Mononuclear cells/μl				
ATG	2628 ± 188	2204 ± 161	2392 ± 201	NS
Non-ATG	2818 ± 194	3253 ± 210	3454 ± 278	NS
P-value†	NS	<0.0005	<0.0005	
T cells (CD3+) (%)				
ATG	51 ± 2 (1353 ± 115)	44 ± 3 (1016 ± 117)	45 ± 2 (1114 ± 120)	<0.01 (NS)
Non-ATG	53 ± 2 (1551 ± 132)	59 ± 2 (1938 ± 156)	57 ± 2 (1982 ± 179)	<0.0005 (<0.05)
P-value	NS (NS)	<0.0005 (<0.0005)	<0.0005 (<0.0005)	
CD4+ T cells (%)				
ATG	40 ± 2 (990 ± 92)	22 ± 2 (460 ± 41)	18 ± 1 (399 ± 33)	<0.0005 (0.002)
Non-ATG	40 ± 1 (1119 ± 89)	37 ± 2 (1237 ± 102)	33 ± 1 (1178 ± 108)	<0.0005 (NS)
P-value	NS (NS)	<0.0005 (<0.0005)	<0.0005 (<0.0005)	
CD8+ T cells (%)				
ATG	21 ± 1 (552 ± 50)	26 ± 2 (607 ± 81)	27 ± 2 (672 ± 83)	NS (NS)
Non-ATG	20 ± 1 (594 ± 58)	23 ± 1 (746 ± 74)	23 ± 1 (796 ± 85)	NS (NS)
P-value	NS (NS)	NS (<0.02)	NS (NS)	
CD4/CD8 ratio				
ATG	1.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	<0.0005
Non-ATG	2.3 ± 0.1	2.0 ± 0.2	1.7 ± 0.1	<0.0005
P-value	NS	<0.0005	<0.0005	
NK cells (CD56+) (%)				
ATG	7 ± 1 (190 ± 24)	5 ± 1 (123 ± 22)	5 ± 1 (135 ± 32)	NS (NS)
Non-ATG	5 ± 1 (179 ± 37)	4 ± 1 (139 ± 16)	4 ± 0 (117 ± 14)	NS (NS)
P-value	NS (NS)	NS (NS)	NS (NS)	
B cells (CD19+) (%)				
ATG	10 ± 1 (266 ± 37)	13 ± 1 (246 ± 23)	9 ± 1 (203 ± 20)	NS (NS)
Non-ATG	9 ± 1 (274 ± 36)	8 ± 1 (249 ± 31)	8 ± 1 (294 ± 66)	NS (NS)
P-value	NS (NS)	0.001 (NS)	NS (NS)	
Monocytes (CD13+) (%)				
ATG	24 ± 2 (613 ± 49)	36 ± 3 (756 ± 61)	33 ± 2 (727 ± 47)	0.01 (NS)
Non-ATG	24 ± 2 (630 ± 52)	24 ± 2 (746 ± 59)	26 ± 2 (864 ± 73)	NS (<0.05)
P-value	NS (NS)	0.001 (NS)	0.02 (NS)	

Values are given as % of mononuclear cells (absolute counts per μl).

P values for comparison of absolute cell counts of mononuclear cell subsets are given in parenthesis.

NS, not significant.

*Wilcoxon signed rank test was used for statistical comparison between pretransplant, 4 and 12 months post-transplant data within the ATG and non-ATG groups, respectively.

†Wilcoxon rank sum test was used for statistical comparison between the ATG and non-ATG patient groups at the time points indicated (pretransplant, 4 and 12 months post-transplant).

group coincided with late acute rejections in four ATG-treated patients. In non-ATG patients, only one late acute rejection occurred which was the result of non-compliance.

With respect to cytokine receptor expression, we found no significant differences of CD25+ B-cell counts and TNF-αR+ or IFN-γR+ monocyte counts, respectively, between the patient groups (Table 3).

Serum sCD30

Pretransplant sCD30 content was comparable between the non-ATG and ATG groups (90 ± 7 vs. 83 ± 4 U/ml). We found no significant association between pretransplant sCD30 and the incidence of acute rejections or 1-year graft survival. ATG induction therapy did not affect sCD30 levels 4 months and 1 year post-transplant [4 months: 51 ± 7 U/ml, non-ATG versus 44 ± 6 U/ml, ATG (NS); 1 year: 46 ± 4 U/ml, non-ATG versus 41 ± 4 U/ml, ATG (NS)].

T-cell helper activity and cytokine secretion

Pretransplant low CD4 helper activity (<10%), enhanced IL-4 (≥2.5 pg/ml) and low IL-10 responses (<100 pg/ml) of CD4 cells predicted a low risk of acute rejection within the first 4 months post-transplant (Fig. 1). Only one of

Table 3. Expression of adhesion molecules, co-stimulatory ligands and cytokine receptors on T cells, B cells and monocytes in renal recipients with or without ATG induction therapy.

	Pretransplant	4 months	1 year	*P*-value*
T cells				
CD54+ (%)(ICAM-1)				
ATG	21 ± 2 (287 ± 42)	29 ± 4 (271 ± 47)	29 ± 4 (265 ± 47)	NS (NS)
Non-ATG	16 ± 2 (265 ± 43)	17 ± 2 (343 ± 55)	14 ± 2 (259 ± 48)	NS (NS)
P-value†	NS (NS)	0.05 (NS)	0.01 (NS)	
CD11a/CD18+ (%) (LFA-1)				
ATG	93 ± 2 (1253 ± 114)	94 ± 2 (954 ± 115)	97 ± 1 (1112 ± 122)	NS (NS)
Non-ATG	93 ± 2 (1421 ± 126)	93 ± 2 (1762 ± 137)	92 ± 2 (1758 ± 162)	NS (NS)
P-value	NS (NS)	NS (<0.0005)	<0.05 (0.001)	
CD2+ (%)				
ATG	98 ± 0 (1325 ± 115)	97 ± 1 (1002 ± 117)	96 ± 2 (1076 ± 122)	NS (NS)
Non-ATG	98 ± 0 (1550 ± 133)	98 ± 0 (1933 ± 158)	99 ± 0 (1995 ± 174)	NS (0.01)
P-value	NS (NS)	NS (<0.0005)	NS (<0.0005)	
CD28+ (%)				
ATG	77 ± 3 (935 ± 87)	60 ± 5 (575 ± 83)	56 ± 5 (556 ± 61)	<0.02 (<0.02)
Non-ATG	74 ± 2 (1122 ± 133)	74 ± 4 (1545 ± 112)	72 ± 3 (1392 ± 145)	NS (NS)
P-value	NS (NS)	NS (0.01)	0.02 (<0.0005)	
B cells				
CD25+ (%)				
ATG	15 ± 2 (33 ± 4)	13 ± 1 (32 ± 4)	20 ± 3 (39 ± 8)	NS (NS)
Non-ATG	17 ± 2 (42 ± 9)	22 ± 3 (45 ± 7)	21 ± 3 (51 ± 13)	NS (NS)
P-value	NS (NS)	NS (NS)	NS (NS)	
Monocytes				
CD54+ (%) (ICAM-1)				
ATG	59 ± 3 (331 ± 36)	67 ± 3 (510 ± 49)	66 ± 4 (453 ± 41)	<0.05 (0.001)
Non-ATG	60 ± 3 (328 ± 35)	59 ± 4 (413 ± 47)	57 ± 3 (462 ± 59)	NS (0.05)
P-value	NS (NS)	<0.05 (NS)	<0.05 (NS)	
CD58+ (%) (LFA-3)				
ATG	63 ± 5 (351 ± 46)	72 ± 5 (581 ± 64)	72 ± 4 (509 ± 44)	NS (0.02)
Non-ATG	63 ± 5 (323 ± 41)	60 ± 5 (424 ± 60)	64 ± 5 (544 ± 71)	NS (NS)
P-value	NS (NS)	<0.05 (NS)	NS (NS)	
CD80+ (%) (B7-1)				
ATG	10 ± 4 (60 ± 12)	5 ± 4 (34 ± 8)	14 ± 5 (83 ± 16)	NS (NS)
Non-ATG	10 ± 4 (60 ± 14)	12 ± 4 (69 ± 15)	16 ± 5 (131 ± 30)	NS (NS)
P-value	NS (NS)	NS (NS)	NS (NS)	
TNF-αR+ (%)				
ATG	9 ± 2 (48 ± 12)	7 ± 1 (55 ± 12)	14 ± 3 (82 ± 13)	NS (<0.05)
Non-ATG	8 ± 1 (42 ± 10)	7 ± 1 (52 ± 9)	14 ± 2 (111 ± 27)	NS (<0.01)
P-value	NS (NS)	NS (NS)	NS (NS)	
IFN-γR+ (%)				
ATG	24 ± 3 (128 ± 23)	25 ± 3 (226 ± 38)	34 ± 4 (242 ± 36)	NS (0.02)
Non-ATG	22 ± 2 (118 ± 21)	23 ± 3 (164 ± 30)	27 ± 4 (224 ± 46)	NS (<0.05)
P-value	NS (NS)	NS (NS)	NS (NS)	

Values are given as % of mononuclear cells (absolute counts per μl).

P values for comparison of absolute cell counts of mononuclear cell subsets are given in parenthesis.

NS, not significant.

*Wilcoxon signed rank test was used for statistical comparison between pretransplant, 4 month and 12 month post-transplant data within the ATG and non-ATG groups, respectively.

†Wilcoxon rank sum test was used for statistical comparison between the ATG and non-ATG patient groups at the time points indicated (pretransplant, 4 and 12 months post-transplant).

the 34 (3%) patients exhibiting at least one of these three low-risk parameters experienced an acute rejection, in contrast to 15 of 48 (31%) patients who did not ($P = 0.001$). Three of the latter patients experienced acute vascular rejection. The predictive value of the three low-risk parameters applies to non-ATG as well as to immuno-

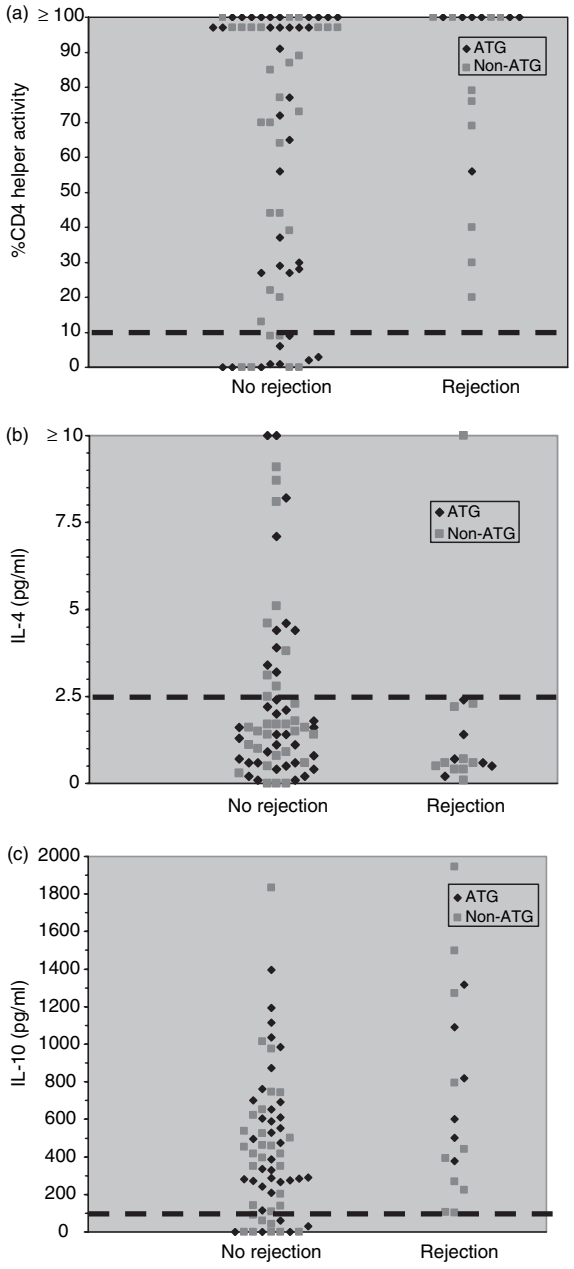

Figure 1 Occurrence of acute rejection episodes during the first 4 months post-transplant in relation to helper activity (a), IL-4 responses (b), and IL-10 responses (c) of CD4+ T cells.

CD4 help <10%, IL-4 responses of ≥2.5 pg/ml, and IL-10 responses of <100 pg/ml were associated with a low risk of acute rejection ($P < 0.05$). None of the 15 patients with pretransplant CD4 helper activity <10%, only one of 20 patients with a pretransplant IL-4 response of ≥2.5 pg/ml, and none of 13 patients with pretransplant IL-10 responses of <100 pg/ml exhibited acute rejection episodes. Each dot represents one patient. Patients who received ATG induction therapy are indicated. As a result of low cell numbers pretransplant, CD4 cell IL-4 and IL-10 responses could not be studied in three of the 84 patients (including one patient who subsequently showed acute vascular rejection).

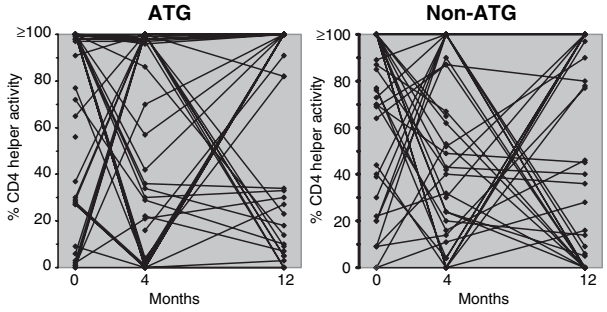

Figure 2 CD4 helper activity pretransplant, 4 and 12 months post-transplant in renal allograft recipients with or without ATG induction therapy (ATG; non-ATG).

No significant differences in CD4 helper activity were found between the ATG and non-ATG groups at any time. ATG induction therapy had no significant effect on CD4 helper activity 4 and 12 months post-transplant when compared with the pretransplant activity (Wilcoxon signed rank test).

logical high-risk ATG patients (Fig. 1). The risk to undergo an acute rejection episode during the first 4 months post-transplant was not significantly different between ATG and non-ATG patients when pretransplant CD4 helper activity was ≥10% [ATG: 20% (7/35); non-ATG: 29% (10/34)], CD4 cell IL-4 responses were <2.5 pg/ml [ATG: 19% (6/31); non-ATG: 31% (9/29)], or CD4 cell IL-10 responses ≥100 pg/ml [ATG: 17% (6/36); non-ATG: 31% (10/32)]. The predictive value of the three low-risk parameters also applies to all three immunosuppressive maintenance regimens given in Table 1 (data not shown).

The ATG induction therapy neither affected CD4 helper activity (Fig. 2) nor IL-4 or IL-10 responses of CD4 cells 4 or 12 months post-transplant (Table 4). In summary, ATG induction therapy induced a profound decrease in CD4+ T-cell counts up to 1 year post-transplant but did not affect helper activity or Th2 responses of CD4+ T cells. The helper activity of 'whole' T cells was also not significantly different in ATG or non-ATG patients (T-helper activity, pretransplant: 109 ± 12%, ATG; 103 ± 12%, non-ATG; 4 months: 86 ± 12%, ATG; 73 ± 12%, non-ATG; 1 year: 88 ± 14%, ATG; 63 ± 13%, non-ATG).

Late acute rejections (5–12 months post-transplant) occurred in four ATG patients (see above). None of them had an acute rejection episode in the first 4 months post-transplant. Two of the four patients showed a low pretransplant CD4 helper activity of <10%. However, CD4 help was increased 4 months post-transplant in all four patients whereas no late acute rejections occurred in 20 patients with a 4-month CD4 help of <10%. The same was true for 4-month CD4 cell IL-10 responses: none of the patients with late acute rejections showed low 4-month responses (<100 pg/ml).

Table 4. Immunoglobulin-secreting cell (ISC) formation and cytokine responses of B cells, T cells and CD4+ T cells in renal recipients with or without ATG induction therapy.

	Pretransplant	4 months	1 year	*P*-value*
SAC-stimulated ISC response (ISC/10^6 B cells)				
ATG	1119 ± 423	1400 ± 301	1760 ± 477	NS
Non-ATG	892 ± 306	1102 ± 321	1890 ± 512	NS
P-value†	NS	NS	NS	
PWM-stimulated ISC response (%)				
ATG	64 ± 12	46 ± 10	63 ± 13	NS
Non-ATG	74 ± 13	34 ± 9	41 ± 11	NS
P-value	NS	NS	NS	
B cell IL-6 response (SAC I) (pg/ml)				
ATG	92 ± 14	209 ± 29	124 ± 16	0.01
Non-ATG	132 ± 25	177 ± 28	163 ± 20	NS
P-value	NS	NS	NS	
B cell IL-6 response (PWM) (pg/ml)				
ATG	26 ± 7	74 ± 16	39 ± 7	0.002
Non-ATG	28 ± 9	68 ± 14	37 ± 7	0.01
P-value	NS	NS	NS	
B-cell IL-10 response (SAC I) (pg/ml)				
ATG	993 ± 196	1986 ± 325	2787 ± 337	<0.01
Non-ATG	1274 ± 248	1883 ± 317	2760 ± 282	<0.005
P-value	NS	NS	NS	
B-cell IL-10 response (PWM) (pg/ml)				
ATG	393 ± 65	495 ± 74	660 ± 75	<0.01
Non-ATG	417 ± 89	391 ± 74	608 ± 65	<0.05
P-value	NS	NS	NS	
T-cell IL-2 response (pg/ml)				
ATG	164 ± 37	107 ± 28	159 ± 26	0.01
Non-ATG	130 ± 26	208 ± 44	296 ± 68	NS
P-value	NS	0.01	NS	
CD4 cell IL-2 response (pg/ml)				
ATG	264 ± 68	127 ± 42	270 ± 63	0.001
Non-ATG	228 ± 61	320 ± 72	585 ± 135	NS
P-value	NS	<0.0005	NS	
T-cell IL-4 response (pg/ml)				
ATG	2.0 ± 0.4	2.0 ± 0.3	2.1 ± 0.2	NS
Non-ATG	1.9 ± 0.4	1.4 ± 0.2	1.6 ± 0.3	NS
P-value	NS	NS	<0.05	
CD4 cell IL-4 response (pg/ml)				
ATG	2.5 ± 0.6	1.6 ± 0.2	2.0 ± 0.3	NS
Non-ATG	2.7 ± 0.8	2.1 ± 0.4	1.4 ± 0.2	NS
P-value	NS	NS	NS	
T-cell IL-10 response (pg/ml)				
ATG	342 ± 52	334 ± 62	367 ± 86	NS
Non-ATG	257 ± 47	404 ± 104	390 ± 51	NS
P-value	NS	NS	NS	
CD4 cell IL-10 response (pg/ml)				
ATG	528 ± 58	610 ± 81	788 ± 152	NS
Non-ATG	439 ± 77	595 ± 133	609 ± 72	NS
P-value	NS	NS	NS	

*Wilcoxon signed rank test was used for statistical comparison between pretransplant, 4 month and 12 month post-transplant data within the ATG and non-ATG groups, respectively. NS, not significant. †Wilcoxon rank sum test was used for statistical comparison between the ATG and non-ATG patient groups at the time points indicated (pretransplant, 4 and 12 months post-transplant).

Coinciding with decreased CD4+ T-cell counts, we found IL-2 responses of both 'whole' T cells and CD4+ T cells to be significantly decreased 4 months post-transplant in ATG-treated patients ($P = 0.01$ and $P < 0.0005$, respectively; Table 4). Even as late as 1 year post-transplant, CD4 cell IL-2 responses were lower in ATG-treated patients but the difference did not reach statistical significance ($P = 0.07$; Table 4). The IL-4 response of 'whole' T cells 1 year post-transplant was marginally elevated in ATG patients ($P = 0.03$; Table 4).

B-cell responses and cytokine secretion

We found no significant effects of ATG induction therapy on ISC formation or B-cell/monocyte-derived IL-6 or IL-10 responses as assayed in SAC I-stimulated B-cell cultures and PWM-stimulated allogeneic cocultures (Table 4).

Discussion

Induction treatment with anti-lymphocyte antibodies reduces the incidence of acute rejection episodes. However, the data in the literature are controversial with respect to an improvement effect on graft survival [25–27]. Side effects of anti-lymphocyte treatment include an increased risk of infections, such as CMV infections, and also an increased risk of malignancies [5–7]. These side effects have to be taken into account when the potential benefit of antilymphocyte induction therapy is evaluated. The investigation of long-term effects of ATG on lymphocyte subset counts and lymphocyte function carried out in the current study is relevant because it might shed light on the mechanism by which ATG exerts its beneficial effect, and also infection and malignancy prone effects. It has to be considered, however, that ATG-treated patients comprise immunological high-risk patients in contrast to non-ATG patients as the primary intention of our prospective study was not to analyze effects of ATG induction therapy. Thus, an increased immunological response as the observed increased adhesion molecule expression on monocytes after ATG induction therapy may be the result of an increased immune responsiveness rather than an effect of ATG whereas long-term suppressed immune responses may definitely be attributed to ATG induction.

The present data confirm previous findings of Müller *et al.* [4] showing that ATG induces a profound long-term decline of the CD4/CD8 ratio with a predominant decline of CD4+ T-cell counts. In contrast to Müller *et al.*, we found no increased regeneration of CD8+ T cells in the first post-transplant year which might be explained by the use of anti-metabolite therapy (Aza or

MMF) in our patients. CD4/CD8 downregulation probably is also occurring in lymphoid tissues, as described by Preville *et al.* [28] in a nonhuman primate model. The initiation of an immune response in lymph nodes would therefore also be affected by prolonged ATG-induced T-cell depletion, and this might in turn have an impact on the severity of infections or the immune response against tumor cells.

We reported previously that increased serum levels of the Th2 activation marker sCD30 before transplantation are associated with graft loss [13] whereas sCD30 in the early post-transplantation period allows the identification of recipients with impending acute rejection [29]. In the current study pretransplant sCD30 levels were not associated with 1-year graft outcome probably because of the low number of graft losses within the first post-transplant year. ATG induction therapy did not show long-term effects on this clinically important Th2 activation marker.

It is an interesting finding of our study that CD4+ T cells were not only diminished numerically but showed a profound decrease in IL-2 secretion up to 4 months post-transplant, coinciding with decreased CD28 expression on T cells which might be an effect of preferential CD28+ T-cell depletion by anti-CD28 antibodies contained in the ATG preparation [30]. A relative enrichment of CD28− CD8+ memory/effector T cells which are less efficiently depleted by ATG treatment, may provide another explanation of the decreased CD28+ T-cell counts [31,32] and might then be expected to result in reduced primary CD8+ T-cell responses to viral antigens and increased T-cell apoptosis [33].

The decreased IL-2 response in the immunological high-risk ATG group might explain the low incidence of acute rejection episodes during the first 4 months post-transplant. It might also explain data of the US renal transplant scientific registry showing that anti-lymphocyte antibody induction therapy was associated with a significant risk of patient death secondary to infectious complications in the first 6 months post-transplant [7]. The increased ATG-induced risk of PTLD [5–7] appears to be primarily the result of inadequate T-cell control (reduced T-cell counts and impaired IL-2 response) of EBV-infected B cells and the concomitantly increased risk of EBV-induced lymphoma formation. No evidence is provided by our data that an increased secretion of the lymphoma growth factors IL-6 and IL-10 may play a role.

Antithymocyte globulin contains polyclonal antibodies directed at different targets, including T and B-cell epitopes and adhesion molecules [30]. The increased adhesion molecule expression on T cells and monocytes of ATG-treated patients as late as 4 months and 1 year after transplantation might be a result of a regulatory process in these immunological high-risk patients. Symptomatic CMV infections during the first post-transplant year, however, had no long-term effects on adhesion molecule expression. The increase in adhesion molecule expression might favor the occurrence of late acute rejections, as observed in four ATG-treated patients in the current series. Elevated CD4 helper activity and increased IL-10 responses 4 months post-transplant in the four patients might also have contributed. In non-ATG patients, only one late acute rejection occurred which was the result of non-compliance.

In HIV-infected patients, we found the decline in CD4+ T-cell counts to be associated with defective helper activity of the remaining CD4+ T cells [22]. Autoantibody formation against CD4 cells was significantly related to the occurrence of CD4 helper defects in these patients [23]. Although ATG contains a series of antibodies directed against CD4+ T-cell epitopes, CD4 helper defects were not induced after ATG therapy, at least as late as 4 or 12 months post-transplant. Thus, ATG induction therapy induced a long-lasting and profound decrease in CD4+ T-cell counts but had no long-term effects on helper activity or Th2 (IL-4, IL-10) responses of CD4+ T cells, which were shown to be predictors of acute rejection. The conclusion of missing long-term effects on CD4 cell help, IL-4 and IL-10 responses may be drawn although ATG-treated patients provided an increased immunological risk profile as these CD4 cell functions – besides comparable values in ATG and non-ATG patients – were also not affected within the ATG group during the 1-year follow-up. We consider this an important result of our study. The predictive value of these parameters was demonstrated both in non-ATG and ATG patients. In accordance with these findings we found ATG not to affect the Th2 activation marker sCD30 which proved to be predictive of graft outcome [13,29]. Thus, ATG induction treatment in immunological high-risk patients does not exert Th2-mediated beneficial long-term effects on graft survival.

As ATG did not affect sCD30 serum levels as late as 4 months and 1 year post-transplant, ATG may not induce long-term expression of the co-stimulatory molecule CD30 on T cells. That might explain why a protective T-cell IL-4 response at least by CD30-mediated signal transduction is not induced [34]. Thus, beneficial long-term effects on the graft by ATG induction may be explained by the long-lasting CD4 cell depletion coinciding with suppression of IL-2 secretion and by decreased CD28 expression on T cells.

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