overhydration. Thus, we would recommend quantification of intrarenal resistance within the kidney graft by using this new and simple parameter—FTI.

There are, however, some limitations of our study. Kidney biopsy was not performed in the majority of patients with delayed graft function, when there was no clinical suspicion of acute rejection or thrombotic microangiopathy. Thus, we are unable to prove that there was no overlapping ATN and subclinical acute rejection in some of the cases.

In conclusion, a discontinuous pattern of Doppler flow spectrum measured in segmental arteries of the kidney graft within the first few days after transplantation is typical for ATN. Moreover, if ATN occurs, a longer duration of ATN in patients with a discontinuous flow pattern is expected. The newly introduced FTI enables better quantitative determination of ATN severity after KTx, especially in patients with a discontinuous flow spectrum.

Conflict of interest statement. None declared.

References


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The effect of rabbit anti-thymocyte globulin induction therapy on regulatory T cells in kidney transplant patients

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Abstract

Background. Prevention of alloreactivity by rabbit antithymocyte globulins (rATG) may not only result from immunodepletion but also from the induction of T cells that control allogeneic immune responses. In the present prospective and controlled study, we investigated the effect of rATG on the frequency, function and phenotype of peripheral immunoregulatory CD4+ T cells in kidney transplant (KTx) patients.

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Methods. After transplantation, 16 patients received ATG-induction therapy and triple therapy consisting of tacrolimus, MMF and steroids. The control group (n = 18) received triple therapy only. By flow cytometry, T cells were analysed for CD25, FoxP3, CD127, CD45RO and CCR7. To study their suppressive capacities, CD25bright T cells were co-cultured with CD25−/dim effector T cells (Teff) in mixed lymphocyte reactions (MLR), stimulated with donor and third party (3P) antigens.

Results. Pre-transplant levels of FoxP3+/CD127−/dim T cells were 6% of CD4+ T cells. One week post-ATG treatment, no measurable numbers of regulatory T cells were present (P < 0.01). After 4 weeks, the cell numbers


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of CD4+FoxP3+CD127−/low T cells slowly reappeared and thereafter remained low (P < 0.01). At 14 weeks, a significant shift towards the CD45RO+CCR7+ (central memory) phenotype within CD4+FoxP3+ T cells was observed (P < 0.01). At 26 weeks, the proliferative alloresponses of the PBMC and CD25−/dim Teff profoundly decreased compared to pre-transplant (P = 0.01 and P = 0.02 respectively), while the regulatory capacity of the CD25bright T cells, of which 90% consisted of FoxP3+CD127−/low T cells, remained unaffected. The CD25bright T cells suppressed the anti-donor (94%) and 3P responses (93%).

Conclusion. Our findings show that rATG therapy does not spare peripheral immunoregulatory T cells in vivo, but after regeneration preserves their suppressive activity.

Keywords: kidney transplantation; memory T cells; patients; rabbit ATG induction therapy; regulatory T cells

Introduction

Thymus-derived natural immunoregulatory CD4+ T cells do not only have important activities in the prevention of autoimmunity [1,2] but also control immune responses towards transplanted organs and tissues [2,3]. They require IL-2 for their homeostasis, function and maintenance and therefore highly express the IL-2 receptor α-chain, CD25 [4]. IL-2 and other members of the IL-2 family that signal via the common γ-chain (CD132) are important for the induction of immunoregulatory CD4+ T cells [5,6]. The markers most generally used to define human immunoregulatory CD4+ T cells are that they constitutively express the forkhead/winged helix transcription factor FoxP3 [7,8], while they do not constitutively express the IL-7 receptor α-chain and are therefore CD127−/low [9]. The majority of the immunoregulatory CD4+ T cells express the CD45RO memory marker, GITR, CTLA-4 [10] and the homing markers CCR7 and CD62L, both necessary for their migration [11]. The proliferation of CD25−/dim effector T cells (Teff) can be inhibited by anergic peripheral CD4+CD25bright T cells either directly in a cell–cell contact-dependent manner [12,13] or via the suppression of the IL-2 and IFN-γ production [14–16]. Other molecular mechanisms of immunoregulatory CD4+ T cell-mediated suppression are through the secretion of IL-10 and TGF-β via killing of the Teff directly by cytolyis [17,18]. It has been shown that immunosuppressive drugs, such as the calcineurin inhibitors (CNI) cyclosporine (CsA) and tacrolimus, negatively affect the frequency and function of CD4+CD25bright T cells [19–21]. However, other agents, for instance the mTOR inhibitors and rabbit anti-thymocyte globulins (rATG), may favour the expansion of the CD4+CD25bright T cells [22–24]. Induction therapy with anti-T-cell depletion strategies as rATG is used to avoid rejection or to minimize the nephrotoxic effect of CNI on immediate graft function [25–27]. Known mechanisms of action by rATG are depletion of immunocompetent cells through complement-dependent lysis or activation-associated apoptosis [28,29] and modulation of several molecules on residual circulating leucocytes that are involved in regulating the leucocyte–endothelium adhesion and leucocyte migration, e.g. the chemokine receptors CXCR4, CCR5 and CCR7 [28,30]. Experimental studies suggested that the immunosuppressive activity of rATG may also result from its effect on CD4+CD25brightFoxP3+ T cells by either the selective sparing of immunoregulatory CD4+ T cells or by the induction and expansion of regulatory T cells [24,31,32]. Therefore, rATG treatment in patients may modulate the immune system and enhance the process leading to hyporesponsiveness towards the allograft.

Here, we characterized the frequency, function and phenotypic characteristics (e.g. FoxP3, CD127, CD45RO and CCR7) of peripheral blood CD4+CD25bright immunoregulatory T cells of KTx patients that received rATG induction therapy combined with triple therapy consisting of tacrolimus, mycophenolate mofetil (MMF) and steroids, prior to transplantation and at 4, 14 and 26 weeks after transplantation. Patients without rATG therapy and only triple therapy served as controls.

Materials and methods

Patients and study design

KTx patients (n = 34) were enrolled in this study. The patients (n = 16) were given one infusion of 2 mg/kg rATG (Thymoglobulin®., Genzyme Corporation, Cambridge, MA) each day at Day 1, 2 and 3 after transplantation followed by a triple therapy maintenance regimen consisting of tacrolimus, MMF and prednisone. MMF and prednisone were given from Day 1 and tacrolimus was given from Day 2 after transplantation. A control non-rATG patient group (n = 18) was given triple therapy alone. Patient characteristics are depicted in Table 1. The patients were part of a feasibility study on a pilot for a future randomized controlled trial and were included between April and August 2007. After informed consent, peripheral blood was drawn pre-transplant and at 4, 14 and 26 weeks after transplantation. The Medical Ethical Commission of the Erasmus Medical Center approved the protocol.

Flow cytometry

EDTA blood was analysed for the presence of T-cell subsets by four-colour flow cytometry using mAbs directly

Table 1. Demographics of kidney transplant patients

<table>
<thead>
<tr>
<th></th>
<th>rATG group</th>
<th>Non-rATG group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients (n)</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td>Recipient gender (M/F)</td>
<td>7/9</td>
<td>8/10</td>
</tr>
<tr>
<td>Recipient age at KTx</td>
<td>53 ± 17a</td>
<td>53 ± 13a</td>
</tr>
<tr>
<td>HLA incompatibilities</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-B MM (number)</td>
<td>0.8 ± 0.8a</td>
<td>1.0 ± 0.8a</td>
</tr>
<tr>
<td>HLA-DR MM (number)</td>
<td>1.2 ± 0.7a</td>
<td>1.3 ± 0.8a</td>
</tr>
<tr>
<td>HLA-DR MM (number)</td>
<td>0.9 ± 0.7a</td>
<td>1.1 ± 0.8a</td>
</tr>
<tr>
<td>Acute rejection (n)</td>
<td>1 (6%)</td>
<td>3 (17%)</td>
</tr>
<tr>
<td>Infections (n)</td>
<td>4 (25%)</td>
<td>2 (11%)</td>
</tr>
</tbody>
</table>

*Mean ± SD.
conjugated to fluorescein (FITC), phycoerythrin (PE), allophycocyanin (APC) or peridinin chlorophyll protein (PerCP). Blood (100 μl) was incubated with CD45-FITC/CD14-PE, IgG1-FITC/IgG2b-PE and CD19-APC. Furthermore, CD3-FITC, CD4-PerCP, CD8-APC and CD3/16/56-FITC/PE were also used (BD Biosciences, San Jose, CA and R&D Systems, Abingdon, UK). After 30-min incubation at RT, red blood cells were lysed. White blood cells were washed twice, and analysed on a flow cytometer (FACSCalibur) using SimulSet and CELL Quest Pro software (BD Biosciences). The number of leukocytes was determined by the cell counter CASY® model TT (Schärfe Systems GmbH, Reutlingen, Germany).

FoxP3 intracellular staining was performed according to the manufacturer’s instructions (FoxP3-APC, clone PCH101, eBiosciences, San Diego, CA). Extracellular staining was conducted prior to intracellular staining with FITC-conjugated CD127, IgG1 or CCR7, PERCP-conjugated CD4 and PE-conjugated CD25 or CD45RO at 4°C for 30 min (BD Biosciences). To discriminate between effector memory (CD45RO+CCR7+), central memory (CD45ROCCR7+) and naïve (CD45ROCCR7+) T cells, CCR7 was combined with CD45RO [33]. Flow cytometric analysis was performed with at least 100 events in the gate.

Isolation of peripheral blood lymphocytes

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation over Ficoll-paque, frozen in a 10% DMSO enriched RPMI 1640 medium (BioWhittaker, Verviers, Belgium) and stored at −140°C.

Isolation of human CD4+CD25bright T cells

PBMC were thawed and resuspended in a 10% human cell medium (HCM) that consisted of RPMI 1640 medium with l-glutamine (Bio Whittaker) supplemented with 10% pooled human serum, 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco BRL). The CD25bright T cells were isolated from PBMC after incubation with anti-CD25 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany), followed by positive selection (POSSELD-program) on the autoMACS (Miltenyi Biotech). The untagged residual fraction consisted of ≥98% of CD25−/dim Teff and was used as the responder population. Both fractions were stained with CD4-PerCP (BD Biosciences) and CD25-PE epitope B (clone M-A251, BD Biosciences). The purity of CD25bright was ≥90%.

Suppression assay

The function of CD4+CD25bright T cells was determined by mixed lymphocyte reactions (MLR) in which the suppressive capacity of CD25bright T cells was measured twice; firstly by their depletion from PBMC, and secondly by their ability to inhibit the proliferative responses to donor and third party (3P) antigens of the CD25−/dim Teff. The CD25−/dim Teff were co-cultured in triplicate with and without CD25bright T cells. Irradiated (40 Gy) donor spleen cells and HLA mismatched (2-2-2) 3P spleen cells were used as stimulator cells (1.105 cells/100 μl) and co-cultured with 5.104 cells/100 μl of a mixture of CD25bright/CD25−/dim at 1:10, 1:20, 1:40 and 1:80 ratios in round-bottom 96-well plates (Nunc, Roskilde, Denmark). Moreover, the CD25bright T cells were co-cultured with irradiated CD25−/dim Teff in the presence of donor and 3P antigens to confirm their anergic state. After 7 days’ incubation at 37°C in a humidified atmosphere of 5% CO2, the proliferation was measured after 3H-thymidine (0.5 μCi/well; Amersham Pharmacia Biotech) incubation during the last 16 h before harvesting. The median counts per minute (cpm) for each triplicate was determined and the level of suppression of the CD25−/dim Teff by CD25bright T cells was calculated and expressed as the percentage inhibition of the Teff.

For the in vitro experiments, pre-Tx samples (n = 3) were stimulated with the mitogen phytohemagglutinin (PHA) in the absence and presence of 1, 10 μg/mL rATG (Thymoglobulin®). Furthermore, CD25−/dim T cells (n = 3) were stimulated with irradiated 2-2-2 HLA-A, B, DR mismatched spleen cells in the presence of 10 μg/ml rATG [24].

Statistical analyses

Statistical analyses were performed using Graphpad Prism (v.4.03). Based on the distribution of the data, we performed non-parametric testing. For determination of the levels of statistical significance, two-sided probability values were used from the Kruskal–Wallis test, the Wilcoxon matched pairs test and the Mann–Whitney U-test. For comparisons within the rATG group over time, the non-parametric Kruskal–Wallis test and the results were validated with the Wilcoxon matched pairs test. To test differences between groups the Mann–Whitney U-test (unpaired measurements) was used. Post hoc analyses were performed using Bonferroni’s test for multiple comparisons. P values ≤0.05 were considered statistically significant.

Results

Patients

We observed acute rejections in three patients of the non-rATG group and in one patient of the rATG group within 26 weeks post-transplantation (post-Tx) (Table 1). The number of infections was not significantly different between both groups. There were no differences in renal function or blood trough levels of tacrolimus between both groups in the first 26 weeks. However, in the non-rATG group, the MMF trough level was significantly higher at 14 weeks after transplantation compared to the rATG group, probably due to adjustments according to leucocyte levels.

Flow cytometry of lymphocyte subsets

After rATG treatment, we observed a significant decrease in CD3+ T cells, CD3+CD16/56+ NK cells, CD8+ T cells, CD4+ T cells, CD4+CD25bright T cells and not in CD19+ B-cells compared to pre-transplant levels in
Fig. 1. Prospective analysis of PBMC and T-cell subpopulations by flow cytometry. Kinetics of different cell types and cell subsets in the rATG-treated patients ($n = 16$, solid line) and non-rATG-treated patients ($n = 18$, dotted line). Data are depicted as mean ± SEM (A) T cells (B) B cells (C) NK cells (D) CD8+ T cells (E) CD4+ T cells (F) CD4+CD25bright T cells of CD4+ T cells (G) CD4+CD25brightFoxP3+ T cells of CD4+ T cells and (H) CD4+CD25brightFoxP3+CD127−/low of CD4+ T cells. Statistically significant differences within the rATG group over time were tested by the Kruskal–Wallis test and validated by the Wilcoxon matched pairs test and differences between the rATG- and non-rATG group were tested with the Mann–Whitney $U$-test.

the rATG group ($P < 0.01$, Figure 1). The magnitude of the decrease in T cells, NK cells, CD4+ T cells, CD4+CD25bright T cells, CD4+CD25brightFoxP3+ T cells and CD4+CD25brightFoxP3+CD127−/low T cells was more outspoken compared to the non-rATG group ($P < 0.01$).

After 4 weeks, the number of all cell types in both groups increased although this recovery occurred more gradually in the rATG group. The number of CD3+ T cells in this group remained significantly lower compared to pre-transplant levels and partial recovery was only 50% of baseline at 26 weeks (Figure 1A). Along with the recovery of the CD4+ T cells, the CD4+CD25bright T cells recovered only to ~30% of baseline at 26 weeks ($P < 0.01$, Figure 1E and F, respectively). To determine the percentage of bona fide regulatory T cells, we further phenotyped the CD4+CD25bright T cells for the regulatory T-cell marker FoxP3. Pre-transplant, the percentage of the CD4+CD25bright T cells that expressed FoxP3 fluctuated between individuals, though the majority of the CD4+CD25bright T cells expressed FoxP3 in both groups, which is in line with the results of Liu et al. (top 10%
Regulatory T cells and rATG induction therapy

Fig. 2. Mixed lymphocyte reactions with PBMC and CD25−/dim Teff. PBMC of rATG-treated patients (n = 6) and non-rATG-treated patients (n = 6) were isolated pre-transplant (dotted bars) and at 14 and 26 weeks and were stimulated in vitro with donor (A) and 3P spleen cells (B) in the MLR. (C) CD25−/dim T cells were stimulated in vitro with donor (C) and 3P (D) antigens. Proliferation is depicted as counts per minute (cpm) after 3H-thymidine incorporation. Error bars represent mean ± SEM.

Suppressive capacity of CD4+CD25brightFoxP3+CD127−/low T cells

The proliferative capacity of the PBMC and the CD25−/dim Teff was analysed in the MLR. At 14 and 26 weeks, the proliferative responses of the allo-activated PBMC population were reduced compared to pre-transplant (Figure 2A and B). This phenomenon was not observed in the non-rATG group. After depletion of the CD25bright T cells, the proliferation of the CD25−/dim Teff in response to allo-antigens showed the same pattern as the PBMC, as their proliferative capacity is affected by rATG (Figure 2C and D). These findings suggest that the anti-donor hyporesponsiveness can be the result of impaired responses by effector T cells and suppressive actions by regulatory T cells.

Therefore, we studied the suppressive capabilities of the CD4+CD25brightFoxP3+CD127−/low T cells at 26 weeks after rATG treatment. Due to the low number of T cells, it was not possible to study the regulatory activities of CD25bright T cells at 4 and 14 weeks after rATG treatment. At 26 weeks, the number of PBMC was sufficient and the function of the CD25bright T cells was measured. After depletion of the CD25bright T cells, the anti-donor and 3P proliferative responses of the CD25−/dim Teff increased, pre- and post-transplant (Figure 3). The CD25bright T cells of post-transplant were anergic in response to donor and 3P antigens and in the presence of irradiated CD25−/dim Teff. Pre- and post-transplant, co-culture of CD25bright T cells and the CD25−/dim Teff resulted in the inhibition of the anti-donor and 3P proliferative responses of the CD25−/dim Teff in a dose-dependent manner (Figure 3). When the percentage of inhibition of the CD25−/dim Teff response to 3P antigens was calculated at different CD25bright :CD25−/dim ratios, this remained proportionally unaltered (Figure 3). Post-transplant, the percentage of inhibition of the anti-donor response at a 1:10 ratio was similar to the non-rATG group (proliferation (cpm))
Fig. 3. Suppressive capacity of regulatory T cells before and after rATG treatment in suppression assays. The ability of CD25<sup>bright</sup> T cells to suppress the anti-donor (grey bars) and 3P responses (dark grey bars) of the CD25<sup>-/dim</sup> Teff was analysed before and after rATG treatment. Proliferation is depicted as counts per minute (cpm) after 3H-thymidine incorporation and the percentage of inhibition of the CD25<sup>-/dim</sup> effector T-cell response (mean) is shown. (A) Suppression assay with PBMC before rATG treatment. The anti-donor and 3P responses of the PBMC and the CD25<sup>-/dim</sup> Teff is given. After co-culture of CD25<sup>-/dim</sup> Teff and CD25<sup>bright</sup> T cells, the anti-donor and 3P responses of the CD25<sup>-/dim</sup> Teff were inhibited in a dose-dependent manner. (B) At 26 weeks after rATG treatment, CD25<sup>-/dim</sup> Teff were also inhibited in a dose-dependent manner. Note that the y-axis is only 10% of the pre-transplant values. Error bars represent mean ± SEM of n = 6.

Fig. 4. Characterization of regulatory T cells that are recovered after rATG treatment. (A) Representative flow cytometric results of CD127<sup>−/low</sup> expression within CD4<sup>+</sup> FoxP3<sup>+</sup> T cells. About 90% of the CD4<sup>+</sup> FoxP3<sup>+</sup> T cells have the CD127<sup>−/low</sup> phenotype. (B) The percentage of CD127<sup>−/low</sup> of CD4<sup>+</sup> FoxP3<sup>+</sup> T cells remained constant both in the rATG and non-rATG group. (C) Evaluation of the kinetics of CD4<sup>+</sup> FoxP3<sup>+</sup> CD127<sup>−/low</sup> T cells before and after transplantation. Absolute cell numbers are shown. CD4<sup>+</sup> FoxP3<sup>+</sup> CD127<sup>−/low</sup> T cells were depleted after rATG treatment (P < 0.01) and showed similar kinetics and pattern as the CD4<sup>+</sup> CD25<sup>bright</sup> T cells. (D) FoxP3<sup>+</sup> CD127<sup>−/low</sup> to FoxP3<sup>+</sup> CD127<sup>int/+</sup> ratios. Data are depicted as mean ± SEM.

0:1 versus 1:10; 20684 ± 11107 versus 3142 ± 2326, mean ± SEM, percentage inhibition: 85%). These results show that though diminished in number, the CD25<sup>bright</sup> T cells have proportionally adequate suppressor activity after rATG treatment, whereas the proliferative capacity of the CD25<sup>-/dim</sup> Teff is significantly affected by rATG.

Phenotypical characterization of CD4<sup>+</sup> FoxP3<sup>+</sup> T cells

We subsequently quantified the CD4<sup>+</sup> regulatory T cells using an approach independent of CD25 expression (Figure 4A). At 1 week after rATG treatment, no measurable numbers of CD4<sup>+</sup> FoxP3<sup>+</sup> CD127<sup>−/low</sup> T cells were present in the circulation. At 4 weeks, 5 out of 16 patients (31%) had a sufficient number of T cells to perform a FoxP3<sup>+</sup> staining. In the rATG group, the percentage of the CD4<sup>+</sup> FoxP3<sup>+</sup> T cells that had the CD127<sup>−/low</sup> phenotype did not change (89% ± 1.0, mean ± SEM) as depicted in Figure 4B, indicating that the recovered CD4<sup>+</sup> FoxP3<sup>+</sup> T cells harboured the phenotype of genuine regulatory T cells. After 4 weeks, along with the depletion of CD4<sup>+</sup> T cells in the rATG group,
the number of patients that had detectable cell numbers of CD4^+FoxP3^+CD127^−/low T cells increased (88%), but in each patient the homeostatic reconstitution occurred slowly (Figure 4C).

**Naive, central memory and effecter memory regulatory T cells**

In the literature it has been described that T cells with the memory phenotype are resistant to immunodepletion by rATG [34] but in our patient cohort we did not measure any T cells immediately after rATG treatment. Hence, the percentage of CD4^+CD45RO^+ profoundly rose above baseline and was higher than the non-rATG group (Figure 5A). The percentage of CD4^+CCR7^+ massively decreased after depletion and was lower than the non-rATG group at 14 and 26 weeks (Figure 5B).

Subsequently, we analysed whether the recovered CD4^+FoxP3^+ T cells after rATG induction were predominantly of the memory phenotype as homeostasis-driven proliferation after immunodepletion may account for an increase in memory CD4^+FoxP3^+ T cells [35]. Therefore, we phenotyped the CD4^+FoxP3^+ T cells for CD45RO and CCR7. The combination of both markers allows a distinction between the central memory and effector memory T cells [33,36,37].

Within the rATG-treated patients in whom we measured CD4^+FoxP3^+ T cells, a shift in the proportion of FoxP3^+ cells expressing CD45RO was observed. Along with the CD4^+ T cells, the proportion of CD4^+FoxP3^+ T cells that expressed CD45RO^+ was higher at 14 and 26 weeks compared to the baseline levels and higher than the non-rATG group (Figure 5C). RATG also influenced the CD4^+FoxP3^+ T cells expressing CCR7, as the proportion of CD4^+FoxP3^+CCR7^+ was lower than in the non-rATG group at 14 and 26 weeks (Figure 5D).

After rATG treatment, significant differences were observed in the composition of the naive (CD45RO^−CCR7^−), central memory (CD45RO^−CCR7^+) and effector memory (CD45RO^+CCR7^−) CD4^+FoxP3^+ T-cell populations. At 14 and 26 weeks, the percentage of naive T cells within the rATG group was lower than pre-transplant and lower than the non-rATG group at 14 and 26 weeks (Figure 5E). The fall in the proportion of naive T cells after rATG treatment was associated with an increase in the proportion of the CD45RO^+ memory pool that was due to an increase of the central memory CD4^+FoxP3^+ T cells (Figure 5F). The percentage of effector memory CD4^+FoxP3^+ T cells remained stable over time, but was higher than in the non-rATG group at 14 and 26 weeks (Figure 5G).

**Discussion**

In the present prospective controlled study, we investigated the effect of rATG induction therapy on the function and phenotype of peripheral CD4^+CD25^bright FoxP3^+CD127^−/low regulatory T cells in KTx patients. Our findings demonstrate that the CD3^+ T cells, CD3^−CD16/56^+ NK cells and CD4^+ T cells, but not CD19^+ B cells are depleted after rATG treatment (Figure 1). The number of T cells steadily recovered to 50% of baseline at 26 weeks. Interestingly, the recovery the number of CD4^+ T cells remained at 30% of baseline. This may be attributed by a slow regeneration and thymic output of CD4^+T cells, which occurs more gradually in the elderly [38].

We found that the regulatory T cells whether defined as CD4^+CD25^bright CD4^+CD25^bright FoxP3^+, CD4^+CD25^bright FoxP3^+CD127^−/low or CD4^+FoxP3^+ CD127^−/low T cells were totally depleted from the peripheral blood after rATG treatment (Figures 1 and 4). Our results are in accordance with Louis et al. who reported that ATG did not specifically spare the CD4^+CD25^bright T cells [29].

In concert with the slow repopulation of the CD4^+ T cells, the CD4^+CD25^bright and CD4^+FoxP3^+CD127^−/low T cells did not fully recover and showed an impaired homeostasis in the first 26 weeks after rATG treatment. We found that the FoxP3^+ expression did not change at the protein level after rATG treatment within 26 weeks after transplantation. Again in line with this, it was described that the FoxP3 mRNA transcripts were not up-regulated within 2 years after transplantation [29]. From these data it can be concluded that regulatory and non-regulatory CD4^+ T cells show the same behaviour after rATG induction therapy. Several studies with other immunodepleting agents as e.g. Campath-1H (anti-CD52) support our and above-mentioned data by showing that the number of CD4^+CD25^brightFoxP3^+ regulatory T cells significantly decreased after treatment and remained very low thereafter, suggesting that this effect is driven by immunodepletion in general [39–41].

After rATG treatment, the proliferative capacity of the PBMC and CD25^−/dim Teff markedly decreased as shown in Figures 2 and 3. Besides general phenomena that account for the hyporesponsiveness as anergy, clonal deletion and ignorance of donor-directed Teff, we have several rATG-specific explanations for the observed hyporesponsiveness. Firstly, the low proliferation can be attributed to a low percentage of T cells among the lymphocyte population and the low number of CD4^+ T cells within the T-cell population as this resulted in an inverse CD4^+ to CD8^+ ratio (Figure 1). The low number of CD4^+ T cells is associated with an inferior T-helper cell response to CD8^+ T cells in comparison with before transplantation. A second explanation is given by Previle et al. who demonstrated in non-human primates that non-depleted CD3^+ and CD4^+ T cells in lymph nodes that were coated by rATG, downregulated CD2, CD3, CD4 and CD8 molecules and had impaired responsiveness in the MLR [42]. Thirdly, other studies reported that T-helper cell function is affected by rATG by impaired co-stimulatory signals delivered by monocytes [43] or by decreased expression of the co-stimulatory molecule CD28 on T cells [44,45], and fourthly, by a post-transcriptional defect of CD25 expression resulting in a reduced IL-2 response, while a normal IL-2 secretion is preserved [43]. Fifthly, as demonstrated by Haidinger and co-workers, the low proliferative capacities of the Teff population could also be due to disruption of the T cell/antigen presenting cell (APC) interface by rATG [46]. Sixthly, rATG triggers lysis of dendritic cells (DCs) [47,48] and impairs their maturation [49] and therefore DCs will not play an active role in the antigen
presentation to T cells. Here, we report another explanation for the hyporesponsiveness of the PBMC, i.e. the partial involvement of functional regulatory T cells. Depletion of the CD25bright population from the PBMC resulted in an increased proliferation, and in co-culture experiments these CD25bright T cells profoundly suppressed the anti-donor and 3P responses after rATG treatment (Figure 3). Therefore, our main finding is that rATG affected the function of
recovered Teff while the suppressive activity of the newly generated regulatory T cells remained proportionally unaltered. The CD4+FoxP3+ T cells measured in the rATG group are probably not regenerated by the thymus as the percentage of naïve T cells profoundly decreased as shown in Figure 5. This suggests that there could be a transient effect on the regenerative capacity of thymus. As we observed a shift in the frequency of CD4+ FoxP3+ CD127−/low T cells towards the central memory phenotype (Figure 5), it is likely that the naïve CD4+FoxP3+ T cells differentiated into the memory CD4+ FoxP3+ T cells. Factors such as donor antigens or rabbit immunoglobulins that act as foreign antigens might contribute to this differentiation. As the increase in the proportion of CD4+ FoxP3+ memory T cells was not observed in the control group, particularly rabbit immunoglobulins must play an important role. To our knowledge, we provide the first evidence that rATG therapy in KTx patients affects the function of CD25−/dim Teff and preserves CD4+ CD25high regulatory T-cell function. As one might expect from experimental studies, the therapeutic effect of rATG in vivo neither arose from the expansion of residual regulatory T cells nor from de novo generation that resulted in an increase above baseline. Most importantly, the regulatory T cells that recovered after rATG treatment were able to effectively govern alloimmune immune responses by effector T cells as before rATG treatment.

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References

Differential effects of immunosuppressive drugs on COX-2 activity in vitro and in kidney transplant patients in vivo

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Abstract

Background. It was hypothesized that calcineurin inhibitors suppress vascular cyclooxygenase (COX)-2 and exert a reciprocal influence on in vivo prostacyclin and thromboxane. This could contribute to cardiovascular morbidity in transplanted patients.

Methods. The ability of immunosuppressive drugs to suppress vascular COX-2 expression in vitro was studied in cultured human vascular smooth muscle cells. Blood and urine samples were collected from 28 renal transplant patients before and 2, 4 and 6 h after intake of immunosuppressives and from 11 controls. ELISA was used to measure (1) plasma 6-keto-PGF1α and TxB2; (2) urine excretion of PGI-M and TxB2; (3) 6-keto-PGF1α in the whole-blood COX-2 assay; and (4) TxB2 in the whole-blood COX-1 assay. Platelet aggregation was measured optically.

Results. COX-2 in cultured vascular smooth muscle cells was suppressed by cyclosporine A (CsA); tacrolimus and rapamycin had no effect. Human renal arteries and vascular smooth muscle expressed calcineurin Aβ and Aγ