Regulatory T cells in kidney transplant recipients: the effect of induction immunosuppression therapy

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Abstract

Background. Regulatory T cells have been suggested to down-regulate the alloimmune response. The aim of this prospective open study was to evaluate the effects of different inductive agents on peripheral blood regulatory T cells in kidney transplant patients and to analyse their association with short-term graft outcome.

Methods. Regulatory and effector T cell numbers in peripheral blood were determined by flow cytometry in 71 prospectively followed kidney transplant recipients at postoperative day 0, 7, 14, 21, 28, 60 and 90. Patients were treated with a calcineurin inhibitor-based triple immunosuppression with polyclonal rabbit anti-thymocyte globulin (rATG, n = 28), basiliximab, the anti-CD25 monoclonal antibody (n = 18) or without induction (controls, n = 25). Flow cytometry data were correlated to rejection incidence.

Results. Compared to controls, CD4+CD25+FoxP3+ regulatory T-cell expansion among CD4+ T cells was noticed in the rATG group at all post-transplant time-points by Day 14 (P < 0.001). A significant decrease in Treg frequency (P < 0.001) and concurrently a transient increase of CD4+CD25low/FoxP3+ population were observed in basiliximab-treated patients 7–60 days post-transplantation. Biopsy-proven acute rejection occurred in 16.7% of controls, 10.7% of the rATG group and in 11.1% of the basiliximab group. Higher CD4+FoxP3+ / CD8+CD45RA−/CD62L− ratios were observed repeatedly in those patients after basiliximab induction who were rejection free (P < 0.01).

Conclusions. In this study, the rATG induction therapy was associated with an expansion of regulatory cells. Sustained high CD4+FoxP3+/Teff ratios were associated with the absence of rejection after basiliximab induction.

Keywords: basiliximab; FoxP3; kidney transplantation; rATG; regulatory T cells

Introduction

Kidney transplants are the most effective method of treating end-stage renal disease. Following transplantation, it is recommended that induction therapies (lymphocyte-depleting agents including rabbit anti-thymocyte globulin (rATG) or interleukin 2 receptor antagonist) be part of the initial immunosuppressive regimens implemented during the treatment of kidney transplant recipients as a means of reducing acute rejection and/or as a means of facilitating the use of reduced dosages of other immunosuppressive regimen components [1].

The mechanism whereby these inductive agents modulate alloimmune responses is not fully understood. Sakaguchi et al. [2] described CD4+CD25+ regulatory T cells in 1995 and Lopez et al. [3] demonstrated that rATG treatment expanded the CD4+CD25+FoxP3+ regulatory T cell (Treg) population in a dose-dependent manner in vitro. These studies uncovered two mechanisms of expansion: conversion of CD4+CD25+ into CD4+CD25+ cells and proliferation of natural CD4+CD25+ T-cell populations.

The currently available interleukin 2 receptor antagonist is basiliximab, a monoclonal antibody with specificity to the IL-2 receptor α chain (IL-2Rα, also known as CD25) present on the surface of activated T-lymphocytes. IL-2/IL-2 receptor (IL-2R) signalling plays a key role in the development and proliferation of antigen-activated T cells that include both effector T cells (Teff) and Tregs [4, 5].

Regulatory T cells have the potential of playing important roles in both the induction and maintenance of tolerance to donor alloantigens in vivo [6]. Circulating Tregs and the presence of FoxP3+ cells in the graft have also been associated with donor-specific hyporesponsiveness in patients after renal transplantation [7]. However, the Treg/Teff ratio could be more important than the number of Tregs present and it has been suggested that a disruption in the Treg/Teff ratio may be linked to graft outcome [8].

The aim of this study was to evaluate the effects of different inductive agents on peripheral regulatory T-cell
blood levels in kidney transplant recipients and to relate these levels to graft rejection.

Materials and methods

Patients and samples

Patients (n = 71) who received renal transplants from deceased donors at our centre between September 2009 and September 2010 were enrolled in this prospective observational study if they signed written informed consent. Flow cytometry was available (weekdays) and patients were not receiving experimental immunosuppression. All patients received immunosuppression according to the centre protocol. Besides the standard triple maintenance therapy that consisted of a calcineurin inhibitor (either tacrolimus or cyclosporine A), mycophenolate mofetil and corticosteroids in the low-risk patients (panel reactive antibodies (PRA) <20%, control group, n = 25), patients received induction therapy in a case of higher immunological risk. Patients with PRAs ≥50% or patients with previous renal transplantation received 1–1.5 mg/kg/day of rATG (rabbit anti-thymocyte globulin, Genzyme Corporation, Cambridge, MA; n = 28) in two to seven doses during the first week post surgery. The median cumulative dose of rATG was 6.7 mg/kg (range 2.0–8.9 mg/kg). Patients with PRA 20–49% or those who received a kidney from an extended criteria donor were treated with 20 mg of basiliximab (Simulect®, Novartis, Basel, Switzerland; n = 18) on Days 0 and 4. Because the number of patients per group was intended to be similar, the time frame for patient recruitment into each group was different.

Except for a difference in the frequency of retransplantation, the mean percentage of PRA and donor age, the clinical characteristics did not differ significantly between the three groups (Table 1). Perioperative blood samples were collected at the following time-points: before transplantation and on Days 7, 14, 21, 28, 60 and 90 post-transplantation. Serum creatinine (SCr) levels, incidence of delayed graft function (DGF), estimated glomerular filtration rate (eGFR) (calculated using the MDRD formula), proteinuria and incidence of acute rejection 3 months post-transplantation were recorded for all patients.

Written informed consent was obtained from all participants in addition to additional signed agreements obtained at the time each biopsy was performed. The study protocol was approved by the Ethics Committee of the Institute for Clinical and Experimental Medicine (No.: 608-08-10).

Table 1. Demographic characteristics at the time of transplantation

<table>
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<tr>
<th></th>
<th>No induction</th>
<th>rATG</th>
<th>Basiliximab</th>
<th>P-value</th>
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<tr>
<td>Number</td>
<td>25</td>
<td>28</td>
<td>18</td>
<td>n.s.</td>
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<tr>
<td>Gender (male/female)</td>
<td>14/11</td>
<td>19/9</td>
<td>9/9</td>
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<tr>
<td>Recipient age (years)</td>
<td>57.9 [27.7; 73.3]</td>
<td>52.9 [21.5; 78.6]</td>
<td>52.9 [25.6; 67.1]</td>
<td>n.s.</td>
</tr>
<tr>
<td>Donor age (years)</td>
<td>52.0 [16.0; 68.0]</td>
<td>46.5 [22.0; 74.0]</td>
<td>63.5 [46.0; 75.0]</td>
<td>&lt;0.001d,e</td>
</tr>
<tr>
<td>1st/2nd and 3rd T × R (n)</td>
<td>25/0</td>
<td>12/16</td>
<td>18/0</td>
<td>&lt;0.0001b</td>
</tr>
<tr>
<td>PRA (%)</td>
<td>4 [0; 36]</td>
<td>64 [0; 96]</td>
<td>0 [0; 44]</td>
<td>&lt;0.0001f,g</td>
</tr>
<tr>
<td>CNI (TAC/CsA) (n)</td>
<td>21/4</td>
<td>28/0</td>
<td>18/0</td>
<td>n.s.</td>
</tr>
<tr>
<td>CIT (hours)*</td>
<td>15.2 [11.0; 20.7]</td>
<td>15.9 [7.7; 22.8]</td>
<td>17.6 [12.4; 21.0]</td>
<td>n.s.</td>
</tr>
<tr>
<td>Dialysis time (years)*</td>
<td>2.0 [0.2; 9.42]</td>
<td>2.2 [0.5; 6.37]</td>
<td>2.1 [0; 4.9]</td>
<td>n.s.</td>
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<td>Cause of renal failure</td>
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<td>TIN: 2</td>
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<td>ANCA vasculitis or lupus nephritis: 0</td>
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<tr>
<td></td>
<td>Other causes: 1</td>
<td>3</td>
<td>1</td>
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</table>

*ANCA, anti-neutrophil cytoplasmic antibodies; CIT, cold ischaemic time; CNI, calcineurin inhibitor; CsA, cyclosporine A; GN, glomerulonephritis; HLA MM, HLA mismatch; PRA, historical panel reactive antibodies, measured every 3 months before transplantation, the highest number was considered in each patient; TAC, tacrolimus; TIN, tubulointerstitial nephritis; T ×, renal transplantation.

A renal graft biopsy was obtained upon clinical presumption of acute rejection, such as an insufficient decline or sudden rise in serum creatinine levels and acute rejection diagnosed histologically according to the Banff ’05 classification [9]. Borderline changes and T-cell-mediated rejection (TCMR) Grades I and IIA were treated by administering 1.5–2 g methylprednisolone, TCMR Grades IIB and III and steroid-resistant TCMR by administering rATG (10 dose; 2 mg/kg on Day 1 and 1 mg/kg for 2–10 days) and antibody-mediated rejection (AMR) by plasma exchange and intravenous immunoglobulin alternately during the 10-day period.

Three patients in the control group who received rATG for treatment of severe (steroid-resistant) acute rejection were excluded from group comparison statistics of flow cytometry data.

Flow cytometry and isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation over Lymphoprep™ (Axis-Shield, Oslo, Norway) from peripheral blood anticoagulated with ethylenediaminetetraacetic acid (EDTA). PBMCs (100 μL, approximately 1 × 10⁶) were labelled with fluoro- chrome-conjugated (PE—phycocyrthin, PC7—phycocyanin-cyanine 7, PC5—phycocyanin-cyanine 5 or FITC—fluorescein) monoclonal antibodies diluted in phosphate-buffered saline–bovine serum albumin buffer for 20 min at room temperature in the dark. The specific antibody panels used consisted of either anti-CD4-PE (clone: 13B8.2), anti-CD8-PC7 (clone: SFC211Thy2D3), anti-CD26L-PC5 (clone: DREG56) and anti-CD45RA-FITC (clone: ALB11) or anti-CD4-PC7 (clone: SFC1124D41), anti-CD25-PC5 (clone: B1.49.9) and anti-CD127-PE (clone: R34.34; Beckman Coulter, Brea, CA).

Intracellular FoxP3 staining of Tregs was performed as described by the manufacturer (Human Regulatory T Cell Staining Kit; ebioscience, San Diego, CA). Extracellular staining of freshly prepared and isolated PBMCs was carried out using anti-CD4-FITC (clone: RPA-T4) and anti-CD25-APC (clone: BC96) antibodies prior to intracellular staining with anti-Foxp3-PE (clone: PCH101). An appropriate isotype control monoclonal antibody (rat IgG2a-PE, cocktail of FITC and APC mouse IgG1) was used to establish the settings used for FoxP3™ Treg analysis.

Following staining, samples were analysed using an FC 500 flow cytometer (Beckman Coulter) and C × P and Kaluza software (Beckman Coulter). Measured T-cell subsets were defined as follows: Tregs as CD4CD25 F Foxp3+ and CD4CD25 CD127- CD8+ Teff as...
CD8<sup>+</sup>CD45RA<sup>-</sup>CD62L<sup>-</sup>. Flow cytometric analyses were performed with at least 100 gated events.

**Statistical analyses**

Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA) and BMDP PC-90 statistical software (BMDP Statistical Software Inc., Los Angeles, CA). Based on the distribution of the data (Shapiro–Wilks’s test for normality), we performed parametric or non-parametric (Mann–Whitney, Kruskal–Wallis) tests. For analysis of categorical variables, chi-square test was used. We performed logarithmic transformation and parametric testing by repeated measures analysis of variance with Bonferroni’s post-test correction when comparing flow cytometry data between the three groups at different time-points. Clinical and flow cytometry data were correlated using the Spearman rank correlation coefficient. Differences were regarded as statistically significant with \( P < 0.05 \). Data are expressed as median values and [min; max] or interquartile range based on their distribution.

**Results**

**Patient and graft survival**

From a total of 71 patients, three patients underwent graft nephrectomy (Day 4, 20 and 58 after transplantation) for renal vein thrombosis, acute haemorrhage after biopsy or primary non-functional graft, respectively; 2 patients died on Days 56 and 80 of sudden death and pulmonal artery embolism, respectively. Two patients withdrew their informed consent to participate in the study at Days 14 and 60.

**Graft function**

The three patient groups examined did not differ in their incidence rates of DGF: controls: 8/25 (32%); the rATG group: 7/28 (25%) and the basiliximab group: 9/28 (32%). No significant differences in the levels of Scr, eGFR and proteinuria were observed, respectively, between treatment groups 90 days post-transplantation: controls, 127 [61; 314] \( \mu \)mol/L, 0.76 [0.19; 1.46] mL/s/1.73 m<sup>2</sup> and 0.22 [0.07; 2.18] g/24 h; rATG, 120 [57; 239] \( \mu \)mol/L, 0.78 [0.29; 1.57] mL/s/1.73 m<sup>2</sup> and 0.18 [0.10; 2.15] g/24 h and basiliximab, 153 [79; 278] \( \mu \)mol/L, 0.59 [0.28; 1.10] mL/s/1.73 m<sup>2</sup> and 0.28 [0.07; 0.49] g/24 h.

**Graft rejection**

Acute rejection occurred during the first 3 months in 4/24 (16.7%) control group patients, 3/28 (10.7%) rATG patients and in 2/18 (11.1%) basiliximab-treated patients (\( P > 0.05 \)). There were another five patients with borderline changes in the control group and seven were treated with basiliximab.

In patients without induction therapy, two patients suffered from early acute TCMR, one patient from acute AMR and one patient had a combined type of rejection. In patients with rATG induction, three patients suffered from AMR and in those with basiliximab, one patient suffered from early acute TCMR and one from combined acute TCMR and AMR.

**CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> Treg cells**

Tregs were defined as CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> cells. When median values were plotted at each time-point examined, we observed a parallel course in the cell numbers present within each group (data not shown). Pre-transplant levels of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> were 4.5 [1.5; 11.2%] and 5.7 [2.0; 11.6%] of the total CD4<sup>+</sup> cells, respectively. The differences observed between Treg populations was due to the narrow gating of the CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cell population carried out with the aim of obtaining maximal purity of this population. CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cell frequencies are further described below.

**CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg levels post rATG treatment**

We observed a depletion in Tregs, as well as in other T-cell subpopulations (data not shown), in patients treated with rATG by Day 7, followed by a slow expansion in Tregs over the 3-month post-transplantation examination period. Treg numbers decreased at Day 7 compared to pre-transplant values and reached 47% of their initial levels by Day 90 (Day 0: 32.3 [1.2; 94.4] \times 10<sup>3</sup>/L; Day 7: 0.2 [0.01; 8.6 \times 10<sup>3</sup>/L; Day 90: 15.3 [4.0; 35.9] \times 10<sup>3</sup>/L; Figure 2A).

Treg frequencies in rATG-treated patients increased at Day 7 compared to pre-transplant values and remained high until Day 90 (Day 0: 3.9 [1.6; 9.1%], Day 7: 7.0 [2.4; 22.3%] and Day 90: 9.6 [2.9; 23.7%]). Treg frequencies were higher at all post-transplant time-points examined compared to controls and were significantly elevated from Day 14 (Day 7: \( P > 0.05 \); Day 14–90: \( P < 0.001 \); Figures 1 and 2A). To evaluate the dose dependence of Treg on Treg frequencies, we correlated the cumulative doses of rATG used and the Treg frequencies observed at each post-transplant time-point. No significant correlations were found.

**CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> levels post basiliximab**

In the basiliximab group, we observed a decrease in both the absolute and relative numbers of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells at Day 7, which remained low until Day 60 and then rose to pre-transplant levels by Day 90 (Day 0: 32.5 [10.5; 133.1] \times 10<sup>3</sup>/L and 5.7 [1.7; 11.2%], Day 7: 1.8 [0.4; 5.1] \times 10<sup>3</sup>/L and 0.2 [0.1; 1.1%], Day 90: 46.8 [7.7; 88.9] \times 10<sup>3</sup>/L and 5.2 [2.4; 10.0%]; Figure 2A). We observed a significantly lower proportion of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells between Days 7–60 (\( P < 0.001 \)) and no differences at Day 90 compared to the control group (Figure 2A).

On the contrary, a CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> population appeared 7 days post-transplantation whose density reached higher levels than pre-transplant values for CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells in both relative and absolute numbers; remaining detectable in all patients until Day 28, in half of the patients by Day 60 and in no patients by Day 90. The disappearance of the CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cell population was in accordance with the reconstitution of the CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T-cell population (Figures 1C and 2B).

The CD4<sup>+</sup>FoxP3<sup>+</sup>/CD8<sup>+</sup>CD45RA<sup>+</sup>CD62L<sup>-</sup> ratio and acute rejection

To define the ratio between regulatory and CD8<sup>+</sup> effector T cells in the whole patient cohort, the CD4<sup>+</sup>FoxP3<sup>+</sup>/T
effector (CD8+CD45RA−CD62L−, Teff) ratio was calculated. The CD4+FoxP3+/Teff ratio values in the rATG group were significantly higher beginning 14 days post-transplantation (P < 0.001) compared to controls. There were not any significant differences in the CD4+FoxP3+/Teff ratio between the basiliximab group and controls (data not shown).

We assessed a possible association of peripheral regulatory and effector T cells with biopsy-proven acute rejection. There were no significant differences in CD4+FoxP3+ frequencies, Teff frequencies and CD4+FoxP3+/Teff ratios between patients without and with acute rejection in control group (without induction therapy) and in those receiving rATG induction at all time-points (P > 0.05; Figure 3). Interestingly, patients without induction therapy who experienced steroid-resistant rejection had higher pre-transplant effector T-cell proportion than the patient with steroid-sensitive rejection (44.9% versus 9.1%).

![Fig. 1. Effect of different inductive agents on Treg cells.](image-url)

(A) Representative dot plots of a patient who received no induction, (B) a patient treated with rATG and (C) a patient treated with basiliximab from Day 0 (left) to 21 (right); CD4+ cells were stained for CD25 and FoxP3 and gated for CD25+FoxP3+ cells. Cells from basiliximab-treated patients were additionally gated for CD25low/FoxP3− expressing cells.
In patients receiving basiliximab induction therapy, the CD4⁺CD25⁺FoxP3⁺ and Teff frequencies and CD4⁺CD25⁺FoxP3⁺/Teff ratios were similar in patients with acute rejection and in those with borderline changes (P > 0.05; Figure 3). Sustained lower Teff frequencies and higher CD4⁺FoxP3⁺/Teff ratios were observed in patients without acute rejection as
compared to patients who experienced either acute rejection or borderline changes (Figure 3). There were significantly lower pre-transplant CD4⁺FoxP3⁺ and Teff frequencies in rejection-free patients compared to patients with either acute rejection or borderline changes (P < 0.05, Figure 3).

Discussion

To the best of our knowledge, this is the first prospective trial that has evaluated the effects of different induction agents on regulatory T cell profiles in the kidneys of transplant patients in vivo. In this study, we analysed the nature of the T-cell subpopulations present and correlated them to clinical data in patients post-renal transplantation that were treated with two different inductive agents: rATG or basiliximab. We demonstrated that rATG increased the proportion of CD4⁺CD25⁺FoxP3⁺ Tregs and that basiliximab caused a decrease in CD4⁺CD25⁺FoxP3⁺ Tregs (and a transient appearance in CD4⁺CD25low/FoxP3⁺ cells). Moreover, the balance between regulatory and effector cells was associated with early acute kidney allograft rejection in basiliximab-treated patients.

Data presented in this study demonstrated that rATG- and basiliximab-treated patients had a decreased acute rejection incidence. This observation is in line with many other studies where rATG induction was associated with reduced acute rejection incidence and improved kidney graft survival [10]. Sufficient evidence suggests that a net benefit effect of anti-IL2-RA treatment compared to placebo (for outcomes in some patients) and a high-quality net benefit in preventing acute rejection [1]. Less is known about the mechanism of action associated with both depletive and non-depletive induction agents.

In this study, we observed in vivo depletion in the CD4⁺CD25⁺FoxP3⁺ cell compartment following rATG treatment as reported previously [11] but the proportion of CD4⁺CD25⁺FoxP3⁺ T cells from CD4⁺ cells was clearly increased. The rise in the relative numbers of Tregs suggested that rATG induced the expansion of these cells as previously described in vitro by Lopez et al. [3] and Sewgobind et al. [12]. Our observations were consistent with recently published data by Gurkan et al. [13] who described both the peripheral expansion and new thymic emigration of T cells with the Treg phenotype induced by 6 mg/kg rATG. In vitro studies [3, 12, 14] demonstrated a dose-dependent increase in the proportion of CD4⁺CD25⁺ T cells using lower rATG concentrations (between 1 and 10 μg/mL or 50 μg/mL) and increased activation (and a decline in the expansion) of CD4⁺CD25⁺ T cells at higher concentrations (50–100 μg/mL or 100 μg/mL) with 10 μg/mL being the optimum concentration for expansion. Serum levels of rATG ranged from 50 to 100 μg/L in clinical conditions [15]. However, levels in lymphoid tissues may be lower, allowing for Treg expansion. Both in vitro [3, 12, 14] and in vivo [11] data demonstrated that rATG affected CD4⁺CD25⁺ T cells numbers but did not affect their function.

Furthermore, we observed a transient rise in CD4⁺CD25low/FoxP3⁺ T cells in patients in the basiliximab group consistent with results described by both Wang et al. [16] and Vondran et al. [17] who demonstrated a down-regulation in CD25 expression following basiliximab induction therapy. Both authors showed that basiliximab did not interfere with the suppressive function of Tregs. Chen et al. [18] showed that CD4⁺FoxP3⁺ T cells could be generated from peripheral CD4⁺CD25⁻ naive responder cells following co-stimulation of the T cell receptor; TGF-β1 combined with Transforming growth factor-β1 signalling in the absence of IL-2. De Goer de Herve et al. [19] suggested that human Treg peripheral cells could be maintained in vivo as a consequence of IL-2Rβ/IL-2 interaction in basiliximab-mediated IL-2Rβ blockage.

Nevertheless, basiliximab has also been shown to hinder CD25 detection using certain fluorochrome-conjugated antibodies that bind a common CD25 epitope [20]. Therefore, reductions in Treg cell populations could be over represented in the presence of a competing anti-CD25 monoclonal antibody used for detection using flow cytometry. Abadja et al. [21] examined the most commonly used anti-CD25 antibody clones used for detection in flow cytometric applications and showed that 6/8 clones interfered with basiliximab thereby resulting in improper staining of the CD25⁺ population. In our study, clones that interfered with basiliximab were used.

It has been broadly suggested that the imbalance in the Treg/Teff ratio may be linked to graft outcome [8]. In our experiment, we confirm this observation in patients after basiliximab induction only. This may be explained by the fact that all acute rejections that occurred in patients after rATG induction were surprisingly antibody mediated. Our previous research suggested differences in immune regulation in AMR and TCMR [22]. Similarly, microarray studies revealed quantitatively specific molecules and pathogenesis-based transcript sets in TCMR and AMR of kidney transplants [23]. These results are in line with the observation of accelerated rejection despite high FoxP3⁺ T-cell infiltration in a rodent model. This experiment suggests effector T cells that have undergone homeostatic proliferation after induced lymphopenia to have augmented functional capabilities and to behave similarly to memory T cells that are resistant to regulation by regulatory T cells [24]. Similarly, the application of rATG was shown to spare the central memory and effector memory T cells in the early post-transplant period [13].

In our study, we observed a trend towards higher pre-transplant effector T-cell proportion in induction-free patients who experienced severe steroid-resistant rejection. This observation may be supported by others where a high proportion of pre-transplant effector T cells were associated with unfavourable outcome [25] but unspacific effector T-cell frequencies may be associated with chronic inflammation that is frequent in dialysed patients. The evaluation of memory T cells by the Enzyme-linked immunosorbent spot (ELISPOT) technique seems to be more feasible to detect alloreactivity at the time of transplantation.

Our measurements showed a parallel course of CD4⁺CD25⁺FoxP3⁺ and CD4⁺CD25⁺CD127⁻ cells which is in agreement with published data of Liu et al. [26], who showed that CD127 expression inversely correlated with FoxP3 expression and that the overwhelming majority of CD4⁺CD25⁺CD127low/FoxP3⁻ cells expressed FoxP3. In contrast, Klein et al. [27] showed that a high frequency
of CD127low cells did not express FoxP3 and, conversely, that there was a high proportion of FOXP3-expressing CD127+ cells in healthy individuals, suggesting that these markers did not represent the same population of Tregs.

A limitation of our study was the inability to define different Treg subtypes as well as to distinguish regulatory T cells from activated T cells transiently expressing FoxP3. This could be possibly overcome by using a more detailed phenotyping approach or by assessing the demethylation status of the FOXP3 gene. However, those experiments were beyond the scope of this study. Similarly, the feasibility of the CD4+CD25+FoxP3+ cell monitoring as biomarker of the efficacy of induction therapy has remained speculative. Firstly, precise and validated tests of alloimmune reactivity and tolerance must be available before the larger biomarker-driven clinical trials can explore its clinical usefulness.

In conclusion, we demonstrated in vivo that rATG increased the proportion of CD4+CD25+FoxP3+ Tregs and that basiliximab caused a transient appearance of CD4+CD25low/FoxP3+ cells along with a decrease in CD4+CD25+FoxP3+ Tregs. Patients treated with induction therapy had a lower incidence of rejection compared to controls. Sustained high CD4+FoxP3+Teff ratios were associated with the absence of acute rejection in patients after basiliximab induction.

Acknowledgements. This study was supported by a grant from the Internal Grant Agency from the Ministry of Health of the Czech Republic No.: IGA MZCR NS 10517-3/2009 and by the Grant Agency of the Czech Republic, grant No.: P301/11/1568. The authors are indebted to Janka Slatinska, Romana Polackova, Martina Ondrakova, Katarina Barcikova, Eva Faberova, Adela Sajdlova and Jelena Skibova for their technical assistance and coordination of the sample collection as well as to the patients and nurses for their cooperation and help.

Conflict of interest statement: O.V. received lecture fees from Genzyme and Novartis and a research grant from Novartis.


Received for publication: 20.6.11; Accepted in revised form: 2.11.11