The impact of induction therapy on the homeostasis and function of regulatory T cells in kidney transplant patients

Anne P. Bouvy, Mariska Klepper, Marcia M.L. Kho, Karin Boer, Michiel G.H. Betjes, Willem Weimar and Carla C. Baan

Department of Internal Medicine, Nephrology and Transplantation, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands

Correspondence and offprint requests to: Anne P. Bouvy; E-mail: a.bouvy@erasmusmc.nl

ABSTRACT

Background. To evaluate the influence of induction therapy on Tregs we investigated their origin, kinetics and function in kidney transplant patients after treatment with T-cell depleting rabbit antithymocyte globulin (rATG) or IL-2 receptor antagonist basiliximab.

Methods. Flow cytometry was used to study thymopoiesis by CD31+ naïve Tregs, homeostatic proliferation by Ki-67+ Tregs and Treg origin by the expression of Helios (nTreg-marker). FACSsorted Tregs were analysed for the demethylation status of the Treg-specific demethylated region (TSDR) of the FoxP3 gene, and Treg-suppressive function.

Results. Differential effects of rATG and basiliximab induction therapies were measured on the repopulation kinetics of Tregs. While decreased absolute numbers of Tregs were found in both study arms, increased percentages of Tregs were found in rATG treated patients and decreased percentages in basiliximab treated patients. In both groups, Treg repopulation was the result of homeostatic proliferation and not of thymopoiesis. At 1 month after rATG and 6 months after basiliximab therapy, high percentages of Ki-67+ Treg were measured, which in the rATG group, was accompanied by low percentages of Ki-67+Helios+ Treg, and by cells with a demethylated TSDR in the FoxP3 gene. After both rATG and basiliximab therapy, repopulated Tregs inhibited proliferation of alloantigen activated T effector cells (Teff).

Conclusions. In kidney transplant patients, repopulation of Treg after rATG and basiliximab therapy is the result of homeostatic proliferation and not of thymopoiesis. These repopulated Treg were functional after both induction strategies; however only after rATG therapy, were increased proportions of Helios− methylated FoxP3 Treg found.

Keywords: homeostatic proliferation, induction therapy, kidney transplantation, regulatory T cells, thymopoiesis

INTRODUCTION

Induction therapy is given to kidney transplant patients to prevent acute rejection and to avoid nephrotoxic effects of high dose calcineurin inhibitors [1, 2]. Commonly used agents are the T-cell depleting rabbit antithymocyte globulin (rATG); thymoglobulin and the non-depleting basiliximab, a monoclonal antibody directed against CD25, the α-chain of the IL-2 receptor (IL-2R) [1–3]. Apart from the effects of rATG and basiliximab to effector T cells, these agents also influence regulatory T cells (Treg), the immunosuppressive counter-mechanism, important for immune regulation [4–13].

Kidney transplant patients treated with rATG induction therapy have increased proportions of circulating Treg [4–7]. The in vivo underlying mechanisms of this phenomenon are not elucidated yet. It is, however, known that Treg, like other T-cell subsets, repopulate after lymphopenia by thymopoiesis, the formation of new T cells called recent thymic emigrants (RTE) and by homeostatic proliferation, the expansion of residual T cells [5, 14, 15]. In addition, there is in vitro evidence that after rATG therapy, functional Treg expand by conversion of conventional T cells into induced Treg [16–21]. Unfortunately, in vivo, conversion of Treg is hard to define. Basiliximab is thought to hamper Treg, as blocking of the IL-2R deprives Treg from IL2 which is important for their homeostasis, survival and...
function [22–25]. It is unclear whether blockade of the IL2R depletes Treg from the circulation, or modifies the CD25-expression [7–12, 26–29].

Tregs can be divided into two subsets; natural Treg (nTreg) and induced Treg (iTreg). nTreg originates directly from the thymus and constitutively express FoxP3; the transcription factor that plays a critical role in their development and function. iTreg, which also express FoxP3, originates in the periphery by the differentiation of conventional T cells into Treg upon exposure to antigen in the presence of tolerogenic cytokines [30, 31]. After rATG induction therapy, it is likely, but still unknown, that in vivo iTreg will contribute to the repopulating Treg pool, as thymopoiesis, the generation of nTreg, decreases with age, and conversion of conventional T cells into iTreg may occur in the presence of donor antigen [16–20, 32]. Both subtypes of Treg have similar phenotypical and functional characteristics and consequently distinction between nTreg and iTreg by the standard markers, CD25, CD127 and FoxP3, is not possible. Recently, a specific DNA methylation pattern within the Treg-specific demethylated region (TSDR) of the FoxP3 gene has been identified that is highly demethylated in nTregs and methylated in iTregs [33]. Moreover, Helios, a member of the Ikaros transcription factor family, has come forward as a marker for nTregs [34].

Here, we assessed how depleting and non-depleting induction therapies influence the mechanism of Treg homeostasis in kidney transplant patients. We determined Treg repopulation mechanisms, thymopoiesis, homeostatic proliferation and Treg conversion along with the analysis of whether nTreg or iTreg are present after induction therapy. These data were completed by functional analysis of the repopulated Treg.

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<th>Table 1. Patient characteristics</th>
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<td><strong>Induction therapy</strong></td>
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<td><strong>Recipient age</strong>&lt;sup&gt;a&lt;/sup&gt; (years)</td>
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<td><strong>Acute rejection</strong>&lt;sup&gt;d&lt;/sup&gt;</td>
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No, number; CMV, cytomegalovirus IgG status (positive or negative) before transplantation; DM, diabetes mellitus. PKD: polycystic kidney disease.
<sup>a</sup>Age in years; median and (range).
<sup>b</sup>Number of patients with 0, 1 or 2 mismatches on DR-locus.
<sup>c</sup>PRA+: current panel reactive antigen: median and (range).
<sup>d</sup>Biopsy proven acute rejection (defined according to the Banff 07 classification [35]) within the first year after transplantation.

**Materials and Methods**

**Patients**

Blood samples were obtained from 33 consecutive kidney transplant patients treated with either rATG (n = 15, Thymoglobuline®, Genzyme/Sanofi, Paris, France) in a dose of 2 mg/kg on Days 1, 2 and 3 after transplantation, or basiliximab (n = 18, Simulect®, Novartis Pharma, Basel, Switzerland), a monoclonal antibody targeting the IL-2R, induction therapy in a dose of 20 mg on Days 0 and 4 after transplantation. Patients received triple maintenance therapy consisting of Tacrolimus (Prograf®, Astellas Pharma Inc., Tokyo, Japan), mycophenolate mofetil (MMF; CellCept®, Hoffmann-La Roche Ltd, Basel, Switzerland) and steroids, which were tapered after 3 months. There were no differences in patient age, gender, cytomegalovirus status and the occurrence of acute rejection episodes between the treatment groups (Table 1). In the rATG group, one patient was lost to follow-up due to graft loss after Month 6 and two patients in the basiliximab group because of rATG anti-rejection therapy at Month 1 and Month 6. The medical ethics committee of the Erasmus Medical Centre approved the study (MEC-2010-022). Blood [heparinized and ethylenediaminetetraacetic acid (EDTA)] was drawn before and at 1, 3, 6 and 12 months after transplantation. Heparimized Lithium blood was used to isolate peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation using standard Ficoll-Paque procedures. Due to a shortage of material we were not able to analyse all parameters for each patient.

**Flow cytometry**

**Absolute numbers of CD4+ T cells.** Absolute counts of CD4+ T cells were determined in EDTA blood using BD MultiTest<sup>TM</sup> 6 color reagent and BD TruCount<sup>TM</sup> tubes (BD Biosciences, San Jose, CA), according to the manufacturer’s instructions. In brief, EDTA blood (50 µL) was added to 20 µL MultiTest reagent in a BD TruCount tube containing a known number of fluorescent beads and incubated for 15 min in the dark at room temperature. After lysisation with BD FACSLysing solution, samples were measured on a FACScanto<sup>TM</sup> (BD Bioscience) and analysed by FACSDiva<sup>TM</sup> software.

**Percentage and absolute number of CD4+CD25+CD127− T cells.** The percentage of CD4+CD25+CD127− T cells was determined in whole blood. Two hundred microlitres of blood was incubated with the following antibodies: CD3-PerCP, CD4-PE, CD25-PE-Cy7 (BD Bioscience) and CD127-FITC (eBioscience, San Diego, CA); hereafter, cells were washed, red blood cells lysed, and fixated using lyse/Fix buffer (BD Bioscience). Next, samples were washed and measured on a FACScantoI and analysed by BD FACSDiva Software. The absolute number of Tregs was determined by multiplying the percentage of CD4+CD25+CD127− T cells with the number of CD4+ T cells.

**Phenotypic characterization of CD4+CD127−FoxP3+ regulatory T cells.** PBMCs were thawed and extracellular
stained with the following fluorochrome-conjugated antibodies: CD3-AmCyan, CD4-APC-H7 (BD Bioscience), CD31-Pe (Biolegend), CD127-PeCy7 and CD45RO-PerCP-Cy5 (BD Bioscience). For intracellular staining with FoxP3-APC, Helios-PB (Biolegend) and Ki-67-FITC (BD Bioscience), the anti-human FoxP3 Staining Kit (eBioscience) was used. As negative control, Fluorescence Minus One controls were performed. A minimum of 100 events per population was set for analysis.

Cytokine production. PBMCs were stimulated in the presence of Brefeldin A (Golgiplug, BD Bioscience) with phorbol myristate acetate (50 ng/mL, Sigma-Aldrich, St. Louis, MO) and Ionomycine (1 µg/mL, Sigma-Aldrich) for 4 h. For determining the production of intracellular cytokines, cells were stained with FoxP3-APC, IL-10-AlexaFluor488 (Biolegend) and IFNγ-PE (BD Bioscience) using the anti-human FoxP3 Staining Kit (eBioscience). Unstimulated cells were used as baseline samples.

Suppression assay

PBMCs were stained with CD3-AmCyan, CD4-PB, CD25-PeCy7, CD127-Pe, 7AAD (BD Bioscience) and cell-sorted for Treg; CD3+CD4+CD25+CD127− and Teff; CD4+CD25−CD127+, using the BD FACSAriaII (BD Bioscience) cell sorter. Purity of sorted Tregs and Teff was >95%. FACSorted Treg were co-cultured in a ratio of 1:5 (Treg:Teff) in a mixed lymphocyte reaction (MLR) consisting of Treg cells stimulated in a 1:1 ratio with γ-irradiated (40 Gy) donor human leucocyte antigen mismatched (2–2–2) third party stimulator cells. After 7 days’ incubation, proliferation was measured as counts per minute (CPM), using Wallac1450 MicroBeta TriLux (PerkinElmer, Groningen, The Netherlands), after 16 h incubation with [3H]-thymidine incorporation (0.5 mCi/well; PerkinElmer, Groningen, The Netherlands), and intracellular cytokines were measured.

The methylation status of the FOXP3 gene

The methylation status of the TSDR of the FoxP3 gene was determined in FACSorted CD3+CD4+CD25+CD127−Treg. Cell pellets were digested with proteinase K and treated with bisulfite using the EZ DNA Methylation-Direct™ Kit (Zymo Research, Irvine, CA). During bisulfite treatment unmethylated cytosines were converted into uracils while methylated cytosines remain unmodified. Hereafter, the TSDR of the FoxP3 gene was amplified by quantitative real-time PCR using the StepOnePlus™ Real-Time PCR System and the TaqMan® Genotyping Master Mix (Applied Biosystems, Foster City, CA). Methylation-specific and demethylation-specific amplification primers and probes were chosen as described by Wieczorek et al. [37]. The percentage of Treg with a demethylated TSDR was calculated using the ratio of amplified demethylated TSDR copies and the sum of amplified methylated and unmethylated TSDR copies. In female patients, the percentage of demethylated TSDR was multiplied by two to correct for the X-linked nature of the FoxP3 gene.

Statistical analysis

Statistical analyses were performed using Graphpad Prism version 5.01. For comparisons within multiple groups the Kruskal–Wallis with Dunn’s multiple comparison test, for comparison between time points or groups, the Mann–Whitney U-test was used. Analysis of contingency tables was conducted using Fisher’s exact test. For relations between parameters, a linear regression model was used and Spearman Rho correlation coefficient ($r_s$) was calculated. P values ≤0.05 were considered statistically significant.

RESULTS

CD4+ T cells and CD4+CD25+CD127− T cells

The absolute number of CD4+ T cells and CD4+CD25+CD127− T cells were determined in the blood of kidney transplant patients treated with rATG or basiliximab induction therapy (Figure 1A–C). As basiliximab hinders the detection of CD25 by certain antibodies, we used clone MA251 detecting CD25epitope B, which does not interfere with basiliximab [38]. At 1 month after rATG induction we observed a significant decrease in the absolute number of both CD4+ T cells and CD4+CD25+CD127− T cells (P < 0.001 versus pre-transplantation, Figure 1B). Thereafter the number of CD4+ T cells and CD4+CD25+CD127− T cells slowly increased but did not reach pre-transplant levels (Figure 1B). Basiliximab treated patients showed no differences in the absolute number of CD4+ T cells while the absolute numbers of CD4+CD25+CD127− T cells decreased at 1 and 3 months (P < 0.01, Figure 1C). At all post-transplant time points, basiliximab treated patients had higher absolute numbers of CD4+ and CD4+CD25+CD127− T cells (Figure 1B) than patients before transplantation (Figure 1D, P < 0.05). In contrast, the proportion of Treg after basiliximab induction therapy was significantly lower for the whole study period (P < 0.01). At Month 3 and Month 6, rATG treated patients had a higher percentage of CD4+CD25+CD127− T cells than basiliximab treated patients (Figure 1D, P < 0.01).

Regulatory T cells after induction therapy and repopulation mechanisms

In addition, Tregs were characterized for the Treg marker FoxP3 and the memory marker CD45RO (Figure 2A). At Month 6, higher percentages of CD4+CD127−FoxP3+ Treg were observed in the rATG group and lower percentages in the basiliximab group (P < 0.05, Figure 2B) than in patients before transplantation. In both patient groups, at all time points, the majority of CD127−FoxP3+ Treg expressed CD45RO, though after rATG treatment the CD45RO expression remained constant, while we found a low percentage of CD4+CD45RO
CD31 expressing RTEs, is an age-related process; the presence of CD31+ naïve T cells decreases with age [39, 40]. This inverse correlation was also present for CD4+CD25+CD127– T cells-pre-NTx and at 1, 3, 6 and 12 months after transplantation in patients treated with rATG (red) and basiliximab (blue) induction therapy. (C) The absolute number of CD4+CD25+CD127– T cells-pre-NTx and at 1, 3, 6 and 12 months after transplantation in patients treated with rATG (red) and basiliximab (blue) induction therapy. (D) The percentage of CD4+CD25+CD127– T cells within the total CD4+ T-cell population-pre-NTx and at 1, 3, 6 and 12 months after rATG (red) and basiliximab (blue) induction therapy, compared with the percentage CD4+CD25+CD127– T cells before transplantation. Data are shown as median ± inter quartile range (IQR), *P < 0.05, **P < 0.01, ***P < 0.001.

Thymopoiesis, the formation of CD31 expressing RTEs, is an age-related process; the presence of CD31+ naïve T cells decreases with age [39, 40]. This inverse correlation was also present for CD31+CD45RO– Treg and age, before and after transplantation (Figure 3B; \( r = -0.6798, \) \( P < 0.05 \) and \( r = -0.5671, \) \( P < 0.001 \)). Consequently, we used CD31 as a marker for RTE-Treg. No differences in the percentage of RTE-Treg were observed (Figure 3C). Homeostatic proliferation of CD4+CD127–FoxP3+ Treg was measured by the expression of Ki-67, an antigen present during the G1–M phase of the cell cycle (Figure 3D) [41]. Patients at 1 month after rATG induction therapy had higher percentages of Ki-67+ Tregs than patients before transplantation (Figure 3E, \( P < 0.001 \)). At 1 month after basiliximab therapy, patients had low percentages of Ki-67+ Tregs. Surprisingly, at 6 months after basiliximab, the percentage of Ki-67+ Tregs was higher than in patients before transplantation (Figure 3E, \( P < 0.05 \)). A comparable expression pattern of Ki-67 was seen by CD45RO+, memory Treg (Figure 3F), while for the CD45RO– naïve Treg, only at 6 months after basiliximab therapy, were high percentages of Ki-67+CD4+CD45RO–CD127–FoxP3+ Treg observed (\( P < 0.05, \) Figure 3G).
Origin of repopulated Tregs

To determine the origin of Tregs present after induction therapy we studied Helios expression by Ki-67+CD4+CD45RO−FoxP3+ Treg (Figure 4A) and the FoxP3 demethylation profile of cell-sorted CD4+CD127−FoxP3+ Treg. At 1 and 12 months after rATG therapy, low percentages of Helios+ Ki-67+CD4+CD45RO+CD127−FoxP3+ Treg were measured (Figure 4B, P < 0.05), suggestive for the generation of iTreg, while after basiliximab therapy the percentage of Helios+ Ki-67+CD4+CD45RO+CD127−FoxP3+ Treg was comparable (Figure 4B). The relation between the expression of Helios and Ki67 in CD45RO+Treg is shown in Figure 4C. From a subset of patients, we had samples at 6–12 months after induction therapy available to study the methylation of TSDR in the FoxP3 gene, where a demethylated TSDR represents an nTreg. Figure 4D shows that after rATG induction therapy, the percentage of cells with a demethylated TSDR in the FOXP3 gene was lower than after basiliximab therapy, suggestive for the induction of iTreg after rATG therapy (P < 0.05).
FIGURE 3: Thymopoiesis and homeostatic proliferation after induction therapy. (A) Representative example of the gating strategy of RTE-Treg; CD4+CD31+CD45RO−CD127−FoxP3+ T cells. (B) Correlation between age and CD4+CD31+CD45RO−CD127−FoxP3+ Treg before and after transplantation; left: before transplantation, $r = -0.6798$, $P < 0.05$, right: after transplantation, $r = -0.5671$, $P < 0.001$. (C) The percentage CD4+CD31+CD45RO−CD127−FoxP3+ Treg in patients before transplantation (pre-NTx) and in patients at 1, 6 or 12 months after rATG or basiliximab induction therapy. (D) Representative example of the gating strategy of the expression of the proliferation marker Ki-67 by CD4+CD127−FoxP3+ Treg and in the naïve, CD45RO−, and memory, CD45RO+, subset. (E) The percentage of Ki-67+ CD4+CD127−FoxP3+ Treg in patients before and in patients at 1, 6 or 12 months after rATG or basiliximab induction therapy. (F) The percentage of Ki-67+ CD4+CD127−FoxP3+ Treg in patients before transplantation and in patients at 1, 6 or 12 months after rATG or basiliximab induction therapy. (G) The percentage of Ki-67+ CD4+CD45RO−CD127−FoxP3+ Treg in patients before transplantation and in patients at 1, 6 or 12 months after rATG or basiliximab induction therapy. Due to a shortage of material we were not able to measure all parameters for all patients. Data are shown as median ± IQR. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. 
Functional capacities of regulatory T cells

We next studied the functional capacities of repopulated Tregs. There was no difference in the IFNγ production capacity by Tregs in patients before and after induction therapy (Figure 5A and B). For the IL-10 production capacity of Tregs, we observed a different pattern. At 1 and 12 months after Tregs received basiliximab therapy, Tregs had a higher IL-10 production capacity than Tregs from patients before transplantation, while only at Month 1 after basiliximab a higher IL-10 production was observed (Figure 5C).

The suppressive capacity of the repopulated Tregs was studied by the classical MLR suppression assay. Teff cells from patients before and at 6–12 months after transplantation were stimulated with donor or third party antigen, in the absence or presence of FACSorted CD4+CD25+CD127− Treg (Treg:Teff = 1:5). Before transplantation, there was no difference in the capacity of Treg to inhibit donor or third party stimulated proliferation of Teff cells; respectively, 6 and 5 out of 11 patients inhibited the proliferative response to donor and third party stimulated cells (Figure 5D). After rATG therapy, Treg from four out of five patients inhibited donor stimulated proliferation but could not control proliferation of third party stimulated Teff cells (Figure 5D and E, P < 0.05). After basiliximab therapy, Tregs comparably inhibited donor and third party stimulated Teff cells in both situations Treg from 4 out 7 patients inhibited Teff proliferation. Due to a large variation in the suppressive capacity of Treg, no statistical differences were found in the percentage of inhibition (Figure 5E).

Rejectors versus non-rejectors

We observed no difference in the occurrence of acute rejection episodes (biopsy proven and defined according to the Banff 07 classification [35]) between rATG and basiliximab treated patients (Table 1). At baseline, no differences were found between rejectors and non-rejectors. No associations were found between rejection and the studied parameters.

DISCUSSION

To define the impact of induction therapy on the immune-suppressive counter mechanism, we here studied different Treg features after rATG and basiliximab induction therapy. In absolute numbers, rATG therapy depleted CD4+CD25+CD127− T cells with an incomplete recovery at 12 months. During repopulation, particularly the first 6 months, high percentages of CD4+CD25+CD127− T cells and CD4+CD127−FoxP3+ T cells were observed. Basiliximab treated patients showed decreased absolute numbers of CD4+CD25+CD127− T cells, which was accompanied by a decreased percentage of CD4+CD25+CD127− T cells, for at least 1 year. For CD4+CD127−FoxP3+ T cells a different observation was made; only at 6 months low percentages of CD4+CD127−FoxP3 T cells were found. After both induction therapies, the Treg pool reconstituted by homeostatic proliferation of Treg with the memory phenotype. However, rATG treated patients had increased percentages of Helios−iTreg, with a demethylated FoxP3 gene, while after basiliximab no changes in Treg phenotype were found. After T-cell depletion, repopulated Treg were able to inhibit donor, but not third party antigen, stimulated Teff cells.

After T-cell depletion, in adult patients, homeostatic proliferation largely contributes to T-cell reconstitution as thymopoiesis is inversely correlated with age [5, 14, 15, 42–44]. However, in patients suffering from T-cell depletion due to chemotherapy, human immunodeficiency virus infection or rATG therapy, increased thymopoiesis has been described [5, 45–47]. We could not confirm these data. An explanation for these findings may be the use of CD31 as RTE-marker, as CD31+ naïve CD4+ T cells in human adults can also be formed by peripheral proliferation [48]. However, as shown earlier by our group, the percentages of Ki-67+CD4+CD127+CD45RO− T cells after rATG therapy are small. Therefore, we speculate that the contribution of proliferating CD31+ naïve Treg is limited. Furthermore, differences in timing [5] or a difference in the extent of end stage renal disease and consequently the duration of uraemia, which influences thymic output [49–51], may underlie the different findings.

As high percentages of memory T cells are found in the circulation after T-cell depletion therapy, Treg repopulation is thought to be the result of homeostatic proliferation of memory FoxP3+ T cells [5, 41, 44]. In addition, in vitro experiments showed that rATG converts CD25− effector T cells into CD25+FoxP3+ suppressor cells [8, 16, 18, 20]. However, Broady et al. [52] reported that these cells were just activated FoxP3+ cells. By analysing the proliferation marker Ki-67, we found, shortly after rATG therapy, indeed high percentages of especially Ki-67+CD45RO+, memory, Treg. In addition, our findings of the nTreg-marker Helios and the methylation status of the FoxP3 gene, as well as the suppressive capacities of Treg, showed that rATG induces iTreg in vivo and that these cells were not activated cells. Though, the Treg repopulation mechanisms do not completely restore the Treg-pool (Figure 1). Phenomena like T-cell exhaustion and deprivation of the cytokine milieu may explain this incomplete recovery [15, 53].

After basiliximab therapy, low numbers of CD4+CD25+CD127− T cells were also observed. This cannot be explained by competition between basiliximab and anti-CD25 antibodies [38]. Some reports state that after basiliximab therapy, CD25+ and CD25+FoxP3+ T cells are depleted, while others state that functional, suppressive, FoxP3+ cells remain in the circulation, albeit with low CD25 expression, caused by modulation or shedding of the CD25 molecule [7, 9, 12, 27–29]. Our findings point to an effect on the CD25 molecule as well, as at 1 month after basiliximab therapy low percentages of CD4+CD25+CD127− T cells, but not of CD4+CD127−FoxP3+ T cells, were found. At 6 months after basiliximab, CD4+CD127−FoxP3+ T cells were affected. This is most likely the net-result of the given immunosuppressive medication, or late apoptotic events triggered by the earlier blockade of the IL2–IL2R pathway.

To determine the origin of Ki-67+Treg, we analysed the expression of Helios, a marker for nTreg [34]. In the rATG group, relatively low percentages of Helios+ proliferating Treg were measured, suggesting a contribution of Helios+iTreg. After basiliximab therapy, we did not observe this phenomenon. Currently, there is some debate about the value of
Helios as a marker for nTreg as recently published studies showed that this marker is also upregulated by Treg and non-Treg after stimulation [54]. In addition, it was found that Helios− and Helios+ Treg co-exist in the nTreg pool [55–58]. Therefore, we complemented our Helios data with the analysis of the demethylation status of the FoxP3 gene. After rATG therapy, the percentage of CD4+CD25+CD127− Tregs with a demethylated TSDR of the Foxp3 gene, i.e. nTreg, decreased confirming our Helios findings.

Among the various mechanisms by which Treg suppress immune responses, the immunoregulatory cytokine IL10 plays a prominent role [13]. After rATG and basiliximab therapy, the IL10 production capacity was higher than in patients before transplantation. When we studied the suppressive capacities of Treg, differences between groups were observed. After rATG therapy the FACsorted CD4+CD25+CD127− Tregs suppressed donor antigen-specific proliferation of Teff cells, whereas proliferation in response to third party antigen was insufficiently inhibited. Tregs of basiliximab treated patients comparably inhibited the proliferation of donor and third party stimulated Teff cells. This difference in suppression between the two treatment groups might be related to the difference found in Helios expression and the demethylation status of the Foxp3 gene; the relative increase in iTreg favoured by rATG treatment. However, as in our study no significant differences were found in the percentage of inhibition between treatment groups and between donor and third party stimulated effector T cells, we can only speculate that these newly formed iTreg, after rATG therapy, have donor-specific immunoregulatory properties.

In conclusion, in kidney transplant patients, repopulation of Treg after rATG and basiliximab therapy is the result of homeostatic proliferation and not of thymopoiesis. These
repopulated Treg were functional after both induction strategies; however, only after rATG therapy, were increased proportions of Helios\textsuperscript{−} methylated FoxP3 Treg found.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest. For laboratory consumables the study was partially financially supported by Sanofi/Genzyme (Paris, France). The authors declare that the results presented in this paper have not been published previously in whole or part, except in abstract form.

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Intensified pharmaceutical care is improving immunosuppressive medication adherence in kidney transplant recipients during the first post-transplant year: a quasi-experimental study

Robert Joost¹, Frank Dörje¹, Judith Schwitulla², Kai-Uwe Eckardt³ and Christian Hugo⁴

¹Pharmacy Department, Erlangen University Hospital, Erlangen, Germany, ²Department of Medical Informatics, Biometry and Epidemiology, Friedrich Alexander University of Erlangen-Nürnberg (FAU), Erlangen, Germany, ³Department of Nephrology and Hypertension, University of Erlangen-Nürnberg (FAU), Erlangen, Germany and ⁴Medizinische Klinik III, Bereich Nephrologie, Universitätsklinikum Carl Gustav Carus, Dresden, Germany

Correspondence and offprint requests to: Christian Hugo; E-mail: christian.hugo@uniklinikum-dresden.de

ABSTRACT

Background. Medication adherence is critical for transplant patients because the consequences of non-adherence can result in allograft loss and may be life threatening.

Methods. A prospective study with 74 renal transplant recipients using a sequential control group design was performed to investigate the impact of a pharmaceutical intensified care programme led by a clinical pharmacist on daily drug adherence during the first year after renal transplantation. Thirty-nine patients of the control group received the already established standardized drug and transplant training, while 35 patients of the intensified care group (ICG) received additional inpatient and outpatient pharmaceutical care and counselling by a dedicated clinical pharmacist. Applied interventions were clustered and classified using the behaviour change technique taxonomy according to Michie. Adherence to immunosuppressive drug therapy was monitored up to 1 year using a medication event monitoring system, pill count (PC), drug holiday (DH) occurrence, Morisky questionnaire and self-report.

Results. Sixty-seven patients (35 of the standard care and 32 of the ICG) were analysed. Implementation of DA was significantly (P = 0.014) improved in patients of the ICG (91%) compared with SCG (75%) during the first year after transplantation. Daily adherence measures were already improved within 30–40 days after start of intensified patient care and continued throughout the study period. Intensified care patients also showed significantly better results for taking adherence (P = 0.006), PC (P = 0.008) and DHs (P = 0.001).

Conclusions. The additional, intensified pharmaceutical care improved patients’ medication adherence remarkably, suggesting that the applied additional care programme has the potential to improve outcomes after organ transplantation.

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