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ORIGINAL ARTICLE

Monotherapy rapamycin allows an increase of CD4^{+} CD25$^{bright+}$ FoxP3^{+} T cells in renal recipients

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Summary

CD4^{+} CD25$^{bright+}$ FoxP3^{+} regulatory T cells (Tregs) may control donor-specific allogeneic responses in kidney transplant recipients. Recent evidence demonstrated that three phenotypical Treg-subsets, naive (CCR7^{+}CD45RO^{-}), central-memory (CCR7^{+}CD45RO^{+}) and effector-memory (CCR7^{-}CD45RO^{+}), are essential for the development and function of antigen-specific suppression in the lymphoid and peripheral tissues. Also, it has been appreciated that Tregs are affected by immunosuppressive agents. In clinical practice, however, the effect of a single drug remains to be determined. Therefore, we analyzed the effect of several immunosuppressive agents on the number, phenotype and function of peripheral Tregs from 46 stable kidney transplant recipients. These patients were converted to monotherapy with tacrolimus (n = 15), rapamycin (n = 17) or mycophenolate mofetil (n = 14). Blood was obtained at inclusion and 6 months thereafter. The number of Tregs increased significantly in patients on monotherapy with rapamycin ($P < 0.001$), which was caused by increased numbers of Tregs with a central-memory and an effector-memory phenotype (both $P < 0.05$). At 6 months after conversion, however, the suppressive function of Tregs did not significantly change in co-cultures stimulated with donor-Ag. Therefore, monotherapy with rapamycin allows the signals that are needed to increase the number of functional Tregs with a memory phenotype, thereby enhancing the potential capacity to regulate donor-specific responses in the lymphoid and the peripheral tissues.

Introduction

Recently, much research has focused on the role of CD4^{+} CD25$^{bright+}$ FoxP3^{+} regulatory T cells (Tregs) in organ transplantation [1–9]. These studies show that donor-specific Tregs may develop after transplantation and actively contribute to donor-specific hyporesponsiveness *in vivo* [1–3,7,9]. Therefore, Tregs may be essential to achieve graft acceptance.

For their development and function, Tregs require dynamic interactions with antigen-presenting cells and T effector cells [10,11]. Increased levels of FoxP3 mRNA were found in the urine of kidney transplant recipients during acute rejection, while Veronese *et al.* demonstrated that FoxP3^{+} cells infiltrated the kidney during acute cellular rejection [12,13]. This implies that Tregs from organ transplant recipients migrate to the lymphoid tissues as well as the graft [12–15]. Insights into the mechanisms and molecules involved in the trafficking of Tregs have recently been provided in patients suffering from ovarian cancer [16]. In line with other studies, the latter study shows that like conventional T cells, Tregs can be distinguished according to their *in vivo* differentiation stage into naive cells (CD45RO^{-}) and memory cells (CD45RO^{+}) [16–24]. Upon activation, naive-Tregs give rise to CD45RO^{+} Tregs [17,25]. Moreover, CD45RO was identified as an important marker for Tregs with potent suppressive capacities [25,26]. Similar to conventional T cells, CD45RO^{+} Tregs can be divided in central-memory Tregs (CM; CCR7^{+}CD45RO^{+}), which migrate to the

draining lymph nodes and effector-memory Tregs (EM; $CCR7^{-}CD45RO^{+}$), which travel to inflamed tissues [16–23]. This suggests that together CM and EM-Tregs control immune responses both in the lymphoid and in the peripheral tissues [27].

At present, transplant patients receive life-long immunosuppressive medication to prevent and treat rejection. At the same time, these drugs may cause a variety of complications such as nephrotoxicity and hypertension and allow opportunistic infections and malignancies to develop [28–30]. Experimental models suggest that immunosuppressive drugs may also affect the development and function of Tregs [31,32]. Here, especially the effect of these drugs on the IL-2 pathway seems important, as this pathway appears to be crucial for the development, homeostasis and function of Tregs [33,34]. Therefore, the effect of calcineurin inhibitors (CNI) that inhibit the production of IL-2 [28], and in contrast, inhibitors of the mammalian target of rapamycin (mTOR) that block the early expansion of alloreactive T cells but spare IL-2 production, has been studied [32,35]. Based upon the results from additional experiments [36–38], it has been suggested that immunosuppressive drugs can affect the composition of Tregs in kidney transplant patients and thereby their migration to draining lymph nodes and the allograft. In clinical practice, however, patients are treated with a combination of drugs and therefore, the effect of any particular single drug remains to be determined.

Here, we prospectively analyzed the number, phenotype (e.g. CD45RO and CCR7) and function of $CD4^{+}$ $CD25^{bright+}$ $FoxP3^{+}$ regulatory T cells from 46 stable kidney transplant recipients on monotherapy. Patients were converted from triple therapy [tacrolimus, mycophenolate mofetil (MMF) and prednisone] to monotherapy tacrolimus, rapamycin or MMF. This provided us with the unique opportunity to determine the individual *in vivo* effect of a single drug on Tregs in kidney transplant patients.

Materials and methods

Subjects

The study was performed at Erasmus MC and all patients provided informed consent according to the rules of our local medical ethics committee. We included 46 stable patients (Table 1), defined as the absence of proteinuria (>0.25 g/l), no increase in serum creatinine levels and no biopsy-proven acute rejection (BPAR) in the last 3 months prior to inclusion. Patients were 2.8 ± 1.4 years after kidney transplantation (KTx) and without malignancies or active infections such as cytomegalovirus (CMV). Fifteen patients were on treatment with statins for hyperlipidemia [39]. As a result, none of our patients presented hyperlipidemia at baseline. From transplantation until study inclusion, immunosuppressive therapy consisted of standard triple therapy with calcineurin inhibitors (CNI), MMF and prednisone. Patients were then randomly assigned to one of the treatment groups with stratification for cadaveric/living transplant and in cohorts of 10. Randomization was carried out by opening a sealed envelope. In arm 1, patients were converted to monotherapy with tacrolimus (n = 15), in arm 2 to rapamycin (n = 17) and in arm 3 to MMF (n = 14). The study medication schedule is shown in Fig. 1. In all arms of treatment, patients also received prednisone for the first month whereafter prednisone was fully withdrawn. The target trough levels for month 6 were 5–10 ng/ml for tacrolimus, 8–12 ng/ml

Table 1. Patient characteristics at inclusion.

Demographics	Arm 1 Tacrolimus (n = 15)	Arm 2 Rapamycin (n = 17)	Arm 3 MMF (n = 14)
Gender (M/F)	8/7	11/6	10/4
Age (years)	50 ± 12	54 ± 10	54 ± 16
Time after KTx (months)	30 ± 13	32 ± 18	39 ± 20
Donor origin living related/deceased	11/4	10/7	9/5
HLA-A/B (mm)	2.3 ± 1.1	2.1 ± 1.2	2.5 ± 0.7
HLA-DR (mm)	1.1 ± 0.7	1.0 ± 0.8	0.9 ± 0.6
Creatinine (μmol/l)	114 ± 22	118 ± 35	114 ± 41
Proteinuria (g/l)	0.08 (0.04–0.14)*	0.07 (0.02–0.21)*	0.08 (0.02–0.23)*
GFR (ml/min/1.73 m^2)	58 ± 14	58 ± 18	62 ± 17
CMV positive pre versus post KTX	8/9	13/14	8/8
BPAR	3	0	3
Statins	5	4	6
Tacrolimus (ng/ml)	6.6 ± 1.8	5.7 ± 2.0	5.5 ± 2.2
MPA (μg/ml)	2.1 ± 1.8	1.9 ± 1.0	2.0 ± 1.0

Mean ± SD; *Median (range); GFR, glomerular filtration rate; CMV, Cytomegalovirus; BPAR, Biopsy Proven Acute Rejection; MPA, mycophenolic acid.

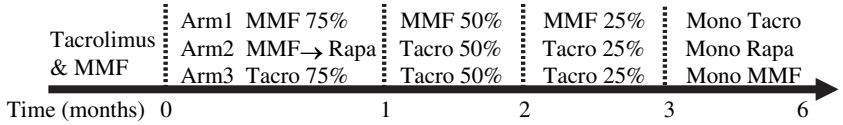

Figure 1 Flow chart for conversion of patients treated with tacrolimus/MMF to monotherapy tacrolimus (arm 1), rapamycin (arm 2) or MMF (arm 3). Full conversion to monotherapy with either drug was achieved at month 3.

for rapamycin and >2 μg/ml for MMF. Peripheral blood samples were obtained at inclusion and 6 months thereafter. There were no significant differences in patient characteristics between the arms of treatment at baseline (Table 1). Also, we included 15 healthy controls (HC), consisting of nine males and six females with a mean age of 51 ± 8 years. These HC were age and gender matched for our patient population.

Flow cytometric analysis

Peripheral blood was collected in heperanized tubes and analyzed for the presence of T-cell subsets by four-color flow cytometry using monoclonal antibody (mAbs) directly conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) or peridinin chlorophyll protein (PerCP). A quantity of 100 μl blood was incubated with 10 μl of the dual mAb combinations CD45-FITC/CD14-PE; IgG_1-FITC/IgG_{2b}-PE; IgG_1-PerCP/IgG_1-APC as isotype control. Further we used the mAb CD3-FITC, CD4-PerCP, CD8-APC and CD25-PE. Also, PBMC were stained for CD3-PerCP, CD4-FITC, CD25-PE (epitope B) and intracellular FoxP3-APC (clone PCH101; eBioscience, San Diego, CA, USA). To determine how Tregs evolve, we added a combination of CD4-PerPC/CD25-PE/CD45RO-APC/CCR7-FITC to 100 μl whole blood. Except for FoxP3, antibodies were purchased from BD Biosciences (San Jose, CA, USA) and R&D Systems (Abingdon, UK). After 30 min of incubation at room temperature, red blood cells were lysed with FACS lysing solution (BD Biosciences) for 10 min. Cells were washed twice, and analyzed on a flow cytometer (FACSCalibur; BD Biosciences) using SimulSet and CELL Quest Pro software (BD Biosciences). To establish an analysis gate that included at least 90% of the lymphocytes, the CD45/CD14 reagent was used. At least 20 000 gated lymphocyte events were acquired from each tube. Cells with a $CCR7^+CD45RO^-$ phenotype were considered to be naive cells, $CCR7^+CD45RO^+$ cells central-memory (CM) and $CCR7^-CD45RO^+$ cells effector-memory (EM).

Isolation of $CD4^+$ $CD25^{bright+}$ $FoxP3^+$ cells

Defrosted patient-PBMC were washed twice and resuspended in 42 μl MACS-buffer/10×10^6 PBMC prepared according to the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). The $CD25^{bright+}$ cells were depleted from PBMC by incubating PBMC with anti-CD25 microbeads (Epitope A; Miltenyi Biotec) followed by a positive selection (POSSELD-program) on the autoMACS® (Miltenyi Biotec). We previously demonstrated that this is an effective method to isolate $CD4^+$ $CD25^{bright+}$ $FoxP3^+$ Tregs [9].

Regulation of donor-Ag stimulated responder cells by $CD25^{bright+}$ cells

A quantity of 5×10^4 defrosted patient-PBMC were incubated with 5×10^4 irradiated (40 Gy) donor PBMC (donor-Ag). Co-cultures were performed in Human Culture Medium (HCM) consisting of RPMI 1640-Dutch Modification (Gibco BRL, Scotland, UK) supplemented with 10% heat-inactivated pooled human serum, 4 mM L-Glutamine (Gibco BRL), 100 IU/ml penicillin (Gibco BRL) and 100 μg/ml streptomycin (Gibco BRL), in triplicate, in a 96-wells round bottom plate for 7 days.

Isolated $CD25^{bright+}$ T cells were added to donor-stimulated patient-PBMC at a 1:10 ratio to determine their regulatory, suppressive capacities [9]. At day 6, ^{3}H-thymidine 0.5 μCi/well was added to the culture and 16 h later, samples were harvested and radioactivity was measured in counts per minute (CPM) using a β-counter (Perkin-Elmer, Oosterhout, the Netherlands).

Inhibition by the $CD25^{bright+}$ cells of the proliferative response was calculated as the percentage of inhibition (%IH).

$$\%\text{IH} = \frac{\text{CPM PBMC} - (\text{CPM PBMC} + \text{CD25}^{\text{bright+}}\text{cells})}{\text{CPM PBMC}} * 100$$

Proliferation of mitogen-stimulated cells

We determined the capacity of PBMC (5×10^4) to proliferate upon stimulation with 1 μg/ml Phytohemagglutinin (PHA; Murex Biotech Ltd, Kent, UK). All cultures were performed in HCM, in triplicate in a 96-wells plate for 3 days. At day 2, ^{3}H-thymidine 0.5 μCi/well was added to the culture and 16 h later, the samples were harvested and radioactivity was counted.

Statistical analysis

Statistics were performed using GraphPad Prism 4.0 (GraphPad Software Inc., La Jolla, CA, USA) or SPSS 11.5 software (SPSS Inc., Chicago, Illinois, USA). To analyze several variables at the same time, linear regression was performed. To analyze differences between before and after treatment with monotherapy, we performed paired analysis (Wilcoxon signed rank test). $*P < 0.05$ and $**P < 0.01$.

Results

Patients

Thirty-nine of the 46 randomized patients (85%) completed the study, while seven (15%) patients were withdrawn as a result of adverse clinical events (rapamycin 2/17; MMF 5/14). Clinical characteristics at 6 months after conversion are summarized in Table 2. Rejection incidence was high in patients on monotherapy MMF (21%), but not on rapamycin or tacrolimus (both 0%). At month 6, the average study drugs trough levels were within or below target levels. The creatinine and protein levels were not different between the arms of treatment at month 6. When compared with baseline, however, the protein level slightly increased in patients treated with rapamycin monotherapy from 0.07 g/l (mean, range: 0.02–0.21 g/l) to 0.12 g/l (mean, range: 0.02–1.56 g/l, $P < 0.01$, Tables 1 and 2). This was clinically relevant as they received treatment with ACE inhibitors. Also, two patients developed hyperlipidemia during the study and were therefore treated with statins [39].

Flow cytometric results

Flow cytometric analysis demonstrated that, irrespective of their treatment, patients were lymphopenic before and after conversion when compared with HC (Table 3). Tregs were defined as the $CD4^+$ $CD25^{bright+}$ T-cell population in combination with slightly less CD4 expression (Fig. 2) [3,9]. At inclusion, the absolute number of Tregs was not different between the arms of treatment (Fig. 3a). At month 6, their number was strongly increased in patients treated with monotherapy rapamycin (Fig. 3a; $P < 0.01$). Also, the intracellular expression of FoxP3 by Tregs was measured. Analysis showed that the number of $FoxP3^+$ Tregs only increased in those patients who had been converted to monotherapy rapamycin (Fig. 3b; $P = 0.05$).

Treatment with monotherapy may also affect the phenotypical characteristics of Tregs. Therefore, we analyzed the expression of CCR7 and CD45 by Tregs to distinguish their naive, EM and CM subsets (Fig. 2). At month 6, the number of naive Tregs seemed to be increased in patients treated with MMF, but because of low statistical power, this was not significant (Fig. 3c). In contrast, the number

Table 2. Patient characteristics 6 months after conversion.

Demographics	Arm 1 Tacrolimus	Arm 2 Rapamycin	Arm 3 MMF
Patient survival	100%	100%	100%
Graft survival	100%	100%	100%
Rejection incidence	0 (0%)	0 (0%)	3 (21%)
Creatinine† (μmol/l)	122 ± 29	121 ± 41	124 ± 29
Proteinuria† (g/l)	0.09 (0.03–2.49)*	0.12 (0.02–1.56)*	0.08 (0.02–0.16)*
GFR† (ml/min/1.73 m^2)	54 ± 14	58 ± 19	56 ± 16
Medication† level	8.6 ± 1.6 (ng/ml)	7.6 ± 1.7 (ng/ml)	5.0 ± 2.1 (μg/ml)

GFR, glomerular filtration rate; Mean ± SD; *Median (range), †Data of patients who completed the study.

Table 3. Flow cytometric results of whole blood.

Cell subsets	Tacrolimus Pre	Tacrolimus Month 6	Rapamycin Pre	Rapamycin Month 6	MMF Pre	MMF Month 6	HC
Lymphocytes	1130 ± 114*	1191 ± 119*	1012 ± 89**	1064 ± 67**	1076 ± 140*	1007 ± 188*	1749 ± 194
$CD3^+$	851 ± 98	861 ± 90	815 ± 77	816 ± 61	794 ± 95	633 ± 159*	1150 ± 159
$CD8^+$	308 ± 56	314 ± 42	375 ± 49	384 ± 52	340 ± 51	211 ± 38	375 ± 75
$CD4^+$	525 ± 62	531 ± 74*	416 ± 50**	412 ± 34**	445 ± 55*	404 ± 134*	772 ± 94

Absolute numbers per μl blood (mean ± SEM), *t*-test was performed for patients versus HC. $*P < 0.05$, $**P < 0.01$.

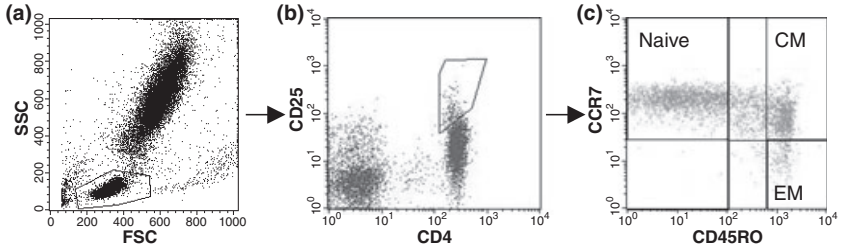

Figure 2 (a) Mononuclear cells were gated with forward scatter (FSC) and sideward scatter (SSC). (b) From the gate in 2A cells with a $CD4^{+}CD25^{bright+}$ phenotype (Treg) were gated. (c) Based on their expression of CCR7 and CD45RO, cells from the gate in 2B were then distinguished into naive Treg ($CCR7^{+}CD45RO^{-}$), central-memory Treg (CM; $CCR7^{+}CD45RO^{+}$) and effector-memory Treg (EM; $CCR7^{-}CD45RO^{+}$).

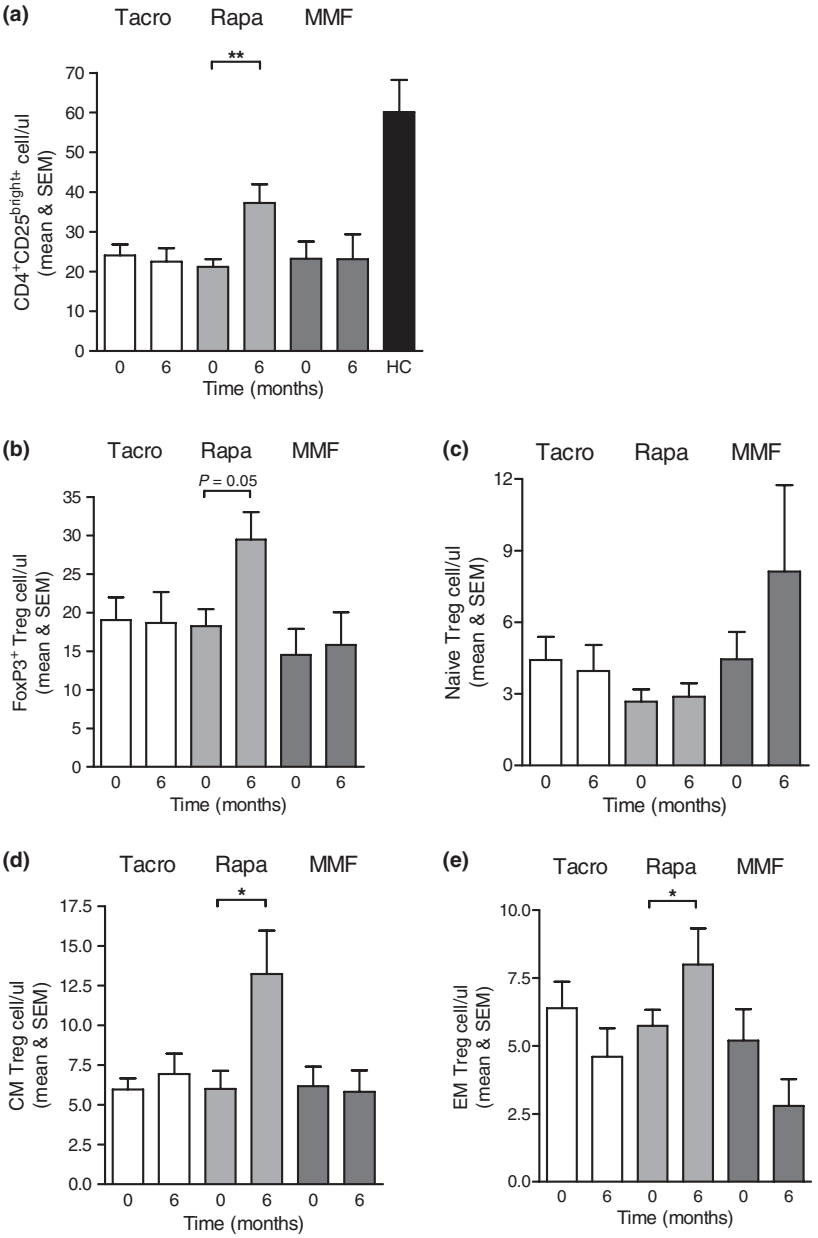

Figure 3 (a–e) At baseline (time 0), the absolute number of Tregs and their subsets were not significantly different between the arms of treatment. (a) The absolute number of Tregs was significantly lower at baseline when compared with healthy controls (HC; all $P < 0.01$). After conversion, the number of Tregs increased significantly in patients treated with rapamycin ($P < 0.01$). (b) The number of Tregs with a $FoxP3^{+}$ phenotype only increased in patients treated with rapamycin ($P = 0.05$). (c) The number of naive-Tregs did not significantly change in either arm of treatment, however, in patients treated with MMF, the number seemed to increase. (d–e) The number of CM and EM-Tregs only increased in patients converted to monotherapy rapamycin (both $P < 0.05$). *$P < 0.05$, **$P < 0.01$.

of CM-Tregs and EM-Tregs increased significantly in patients treated with monotherapy rapamycin (Fig. 3d,e; both $P < 0.05$), while in patients treated with monotherapy tacrolimus or MMF the number of EM-Tregs decreased (Fig. 3e).

The suppressive function of CD25$^{bright+}$ cells

Proliferation of PBMC to the mitogen PHA was >30000 CPM at all tested time points (data not shown).

The suppressive capacity of the isolated CD4$^+$CD25$^{bright+}$ FoxP3$^+$ cells was determined in co-culture experiments with donor-stimulated patient-PBMC. Before conversion, the average capacity of CD4$^+$ CD25$^{bright+}$ FoxP3$^+$ cells to suppress the anti-donor response was 37% (range: 1–85%, Fig. 4). At 6 months after conversion, the capacity of the CD4$^+$CD25$^{bright+}$FoxP3$^+$ cells to suppress the anti-donor response of PBMC from patients on monotherapy with rapamycin, tacrolimus or MMF, was comparable to the percentage measured before conversion and not different between the various therapies (Fig. 4).

Multivariate analysis

In a multivariate analysis, the factors gender, recipient age, time after KTx, origin of donor kidney, HLA mismatch of donor versus recipient, treatment with statins before and during the study period, history of BPAR, and CMV infection after KTx were not associated with the number of Tregs, their phenotypical subsets or suppressive function over time.

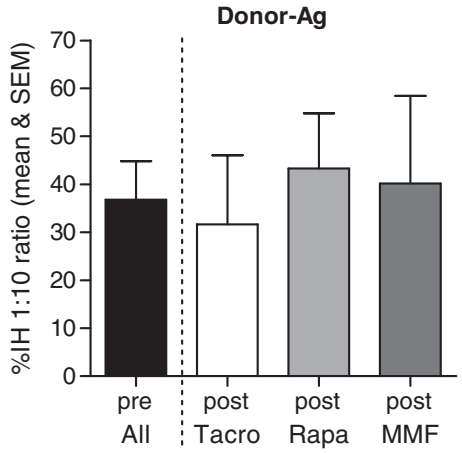

Figure 4 Data are representative for experiments from 19 patients (Tacro $n = 6$, Rapa $n = 7$, MMF $n = 6$). At 6 months after conversion, the capacity of the CD4$^+$ CD25$^{bright+}$ FoxP3$^+$ cells to suppress the anti-donor response of PBMC from patients on monotherapy with tacrolimus, rapamycin or MMF was comparable to the percentage measured before conversion, and not different between the various arms of treatment (ANOVA, $P = 0.82$).

Discussion

We prospectively analyzed peripheral Tregs from 46 renal recipients to investigate whether in a clinical transplant setting monotherapy with tacrolimus, rapamycin or MMF affects the number, phenotype and function of these cells. This is the first study that allows conclusions on the *in vivo* effect of a single immunosuppressive drug on CD4$^+$ CD25$^{bright+}$ FoxP3$^+$ regulatory T cells.

We demonstrated that in kidney transplant recipients Tregs can be distinguished into naive and memory populations and that based on their expression of CCR7 the memory Treg-compartment contains a CM population and an EM population [16].

Before conversion, the low number of peripheral Tregs might have resulted from treatment with tacrolimus and MMF. Tacrolimus has been shown to decrease the thymical output of Tregs in mice, while MMF inhibited Treg-expansion [40,41]. Further analysis revealed that within the Treg compartment the number of naive, CM and EM-Tregs was low. Naive-Tregs give rise to potent memory-Tregs *in vitro* and their low number before conversion suggests little capacity to generate memory-Tregs [25]. We are aware, however, that the number of naive-Tregs decreases with age and therefore the latter effect may be less substantial in our patients [20]. Another explanation for the low number of circulating Tregs at baseline might be their increased migration to the lymph nodes and transplanted kidney [12,13,15].

Analysis of patients that completed the study showed that the number of CD4$^+$ CD25$^{bright+}$ FoxP3$^+$ cells only increased in patients treated with monotherapy rapamycin. The differential effects of immunosuppressive drugs are probably best explained by their different mechanisms of action. First, tacrolimus almost completely inhibits the production of IL-2 by effector T cells [28,34,42,43]. MMF does not directly inhibit the production of IL-2 but by preventing cell-division and down regulating the receptor for IL-2 (CD25), it may severely affect the number of Tregs [28,32]. Rapamycin does not prevent the production of IL-2 nor does it affect the expression of CD25 [28,32]. Furthermore, its lack of effect on Tregs may be explained by the almost complete absence of the signaling pathway leading to mTOR [44].

The observed increase in peripheral Tregs from patients treated with rapamycin was because of an enlargement of the CM and EM compartment. Whether the number of naive-Tregs remains low by little output from the thymus, or by rapid differentiation into memory-Tregs remains to be determined.

Recently it was shown, that CM- and EM-Tregs do not seem to differ in their regulatory capacity [16]. Their differential expression of CCR7, however, directs them to the lymphoid or the peripheral tissues to perform their suppressive function [16,17]. Therefore, the strong increase in the number of CM- as well as EM-Tregs in patients treated with monotherapy rapamycin, suggests that when compared with baseline, their capacity to control allogeneic responses is enhanced both in the lymphoid and the peripheral tissues.

To determine the effect of conversion to either monotherapy with tacrolimus, rapamycin or MMF on the function of $CD4^+$ $CD25^{bright+}$ $FoxP3^+$ cells, we analyzed their suppressive capacity in co-culture experiments. At 6 months after conversion to monotherapy with tacrolimus, rapamycin or MMF, the regulatory activities of $CD4^+$ $CD25^{bright+}$ $FoxP3^+$ cells from these three patient groups were comparable. Therefore, this study suggests that within the time frame of 6 months, neither monotherapy with tacrolimus, rapamycin or MMF has a beneficial effect on the suppressive function per cell. Yet, the higher number of $CD4^+$ $CD25^{bright+}$ $FoxP3^+$ cells in combination with unaltered function suggests a higher potential of Tregs in patients on rapamycin in comparison to patients on tacrolimus or MMF.

In conclusion, this study demonstrated that monotherapy rapamycin facilitates the signals that are needed to increase the number of functional peripheral Tregs with a memory phenotype that have the potential to home to the lymphoid and the peripheral tissues.

Authorship

TKH: performed/designed the research and study, analyzed the data and wrote the paper. JHLV: performed research and wrote the paper. MK: performed research. EVG: performed and designed the study. AG: performed the study. WS: performed research. CCB: designed the study and corrected the paper. WW: designed the study and corrected the paper.

References

1. Game DS, Hernandez-Fuentes MP, Chaudhry AN, Lechler RI. CD4+CD25+ regulatory T cells do not significantly contribute to direct pathway hyporesponsiveness in stable renal transplant patients. *J Am Soc Nephrol* 2003; **14**: 1652.
2. Li Y, Koshiba T, Yoshizawa A, *et al.* Analyses of peripheral blood mononuclear cells in operational tolerance after pediatric living donor liver transplantation. *Am J Transplant* 2004; **4**: 2118.
3. Velthuis JH, Mol WM, Weimar W, Baan CC. CD4+CD25bright+ regulatory T cells can mediate donor nonreactivity in long-term immunosuppressed kidney allograft patients. *Am J Transplant* 2006; **6**: 2955.
4. Braudeau C, Racape M, Giral M, *et al.* Variation in numbers of CD4+CD25highFOXP3+ T cells with normal immuno-regulatory properties in long-term graft outcome. *Transpl Int* 2007; **20**: 845.
5. Kingsley CI, Nadig SN, Wood KJ. Transplantation tolerance: lessons from experimental rodent models. *Transpl Int* 2007; **20**: 828.
6. Sanchez-Fueyo A, Domenig CM, Mariat C, *et al.* Influence of direct and indirect allorecognition pathways on CD4+CD25+ regulatory T-cell function in transplantation. *Transpl Int* 2007; **20**: 534.
7. Bestard O, Cruzado JM, Mestre M, *et al.* Achieving donor-specific hyporesponsiveness is associated with FOXP3+ regulatory T cell recruitment in human renal allograft infiltrates. *J Immunol* 2007; **179**: 4901.
8. Akl A, Jones ND, Rogers N, *et al.* An investigation to assess the potential of CD25highCD4+ T cells to regulate responses to donor alloantigens in clinically stable renal transplant recipients. *Transpl Int* 2008; **21**: 65.
9. Hendrikx TK, van Gurp EA, Mol WM, *et al.* End-stage renal failure and regulatory activities of CD4+CD25bright+FoxP3+ T-cells. *Nephrol Dial Transplant* 2009; in press.
10. Lohr J, Knoechel B, Abbas AK. Regulatory T cells in the periphery. *Immunol Rev* 2006; **212**: 149.
11. Ochando JC, Homma C, Yang Y, *et al.* Alloantigen-presenting plasmacytoid dendritic cells mediate tolerance to vascularized grafts. *Nat Immunol* 2006; **7**: 652.
12. Muthukumar T, Dadhania D, Ding R, *et al.* Messenger RNA for FOXP3 in the urine of renal-allograft recipients. *N Engl J Med* 2005; **353**: 2342.
13. Veronese F, Rotman S, Smith RN, *et al.* Pathological and clinical correlates of FOXP3+ cells in renal allografts during acute rejection. *Am J Transplant* 2007; **7**: 914.
14. Walker LS, Chodos A, Eggena M, *et al.* Antigen-dependent proliferation of CD4+ CD25+ regulatory T cells in vivo. *J Exp Med* 2003; **198**: 249.
15. Dijke IE, Velthuis JH, Caliskan K, *et al.* Intragraft FOXP3 mRNA expression reflects antidonor immune reactivity in cardiac allograft patients. *Transplantation* 2007; **83**: 1477.
16. Tosello V, Odunsi K, Souleimanian NE, *et al.* Differential expression of CCR7 defines two distinct subsets of human memory CD4+CD25+ Tregs. *Clin Immunol* 2008; **126**: 291.
17. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol* 2004; **22**: 745.
18. Ochando JC, Yopp AC, Yang Y, *et al.* Lymph node occupancy is required for the peripheral development of alloantigen-specific Foxp3+ regulatory T cells. *J Immunol* 2005; **174**: 6993.

19. Siegmund K, Feuerer M, Siewert C, *et al.* Migration matters: regulatory T-cell compartmentalization determines suppressive activity in vivo. *Blood* 2005; **106**: 3097.
20. Seddiki N, Santner-Nanan B, Tangye SG, *et al.* Persistence of naive CD45RA+ regulatory T cells in adult life. *Blood* 2006; **107**: 2830.
21. Lee JH, Kang SG, Kim CH. FoxP3+ T cells undergo conventional first switch to lymphoid tissue homing receptors in thymus but accelerated second switch to nonlymphoid tissue homing receptors in secondary lymphoid tissues. *J Immunol* 2007; **178**: 301.
22. Siewert C, Menning A, Dudda J, *et al.* Induction of organ-selective CD4+ regulatory T cell homing. *Eur J Immunol* 2007; **37**: 978.
23. Menning A, Hopken UE, Siegmund K, *et al.* Distinctive role of CCR7 in migration and functional activity of naive- and effector/memory-like Treg subsets. *Eur J Immunol* 2007; **37**: 1575.
24. Valmori D, Merlo A, Souleimanian NE, *et al.* A peripheral circulating compartment of natural naive CD4 Tregs. *J Clin Invest* 2005; **115**: 1953.
25. Hoffmann P, Eder R, Boeld TJ, *et al.* Only the CD45RA+ subpopulation of CD4+CD25high T cells gives rise to homogeneous regulatory T-cell lines upon in vitro expansion. *Blood* 2006; **108**: 4260.
26. Jonuleit H, Schmitt E, Stassen M, *et al.* Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. *J Exp Med* 2001; **193**: 1285.
27. Kang SM, Tang Q, Bluestone JA. CD4+CD25+ regulatory T cells in transplantation: progress, challenges and prospects. *Am J Transplant* 2007; **7**: 1457.
28. Halloran PF. Immunosuppressive drugs for kidney transplantation. *N Engl J Med* 2004; **351**: 2715.
29. Lopez MM, Valenzuela JE, Alvarez FC, *et al.* Long-term problems related to immunosuppression. *Transpl Immunol* 2006; **17**: 31.
30. Gutierrez-Dalmau A, Campistol JM. Immunosuppressive therapy and malignancy in organ transplant recipients: a systematic review. *Drugs* 2007; **67**: 1167.
31. Golshayan D, Buhler L, Lechler RI, Pascual M. From current immunosuppressive strategies to clinical tolerance of allografts. *Transpl Int* 2007; **20**: 12.
32. Demirkiran A, Hendrikx TK, Baan CC, van der Laan LJ. Impact of immunosuppressive drugs on CD4+CD25+FOXP3+ regulatory T cells: does in vitro evidence translate to the clinical setting? *Transplantation* 2008; **85**: 783.
33. Thornton AM, Donovan EE, Piccirillo CA, Shevach EM. Cutting edge: IL-2 is critically required for the in vitro activation of CD4+CD25+ T cell suppressor function. *J Immunol* 2004; **172**: 6519.
34. Maloy KJ, Powrie F. Fueling regulation: IL-2 keeps CD4+ Treg cells fit. *Nat Immunol* 2005; **6**: 1071.
35. Battaglia M, Stabilini A, Roncarolo MG. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood* 2005; **105**: 4743.
36. Koprak S, Matheravidathu S, Springer M, *et al.* Down-regulation of cell surface CXCR6 expression during T cell activation is predominantly mediated by calcineurin. *Cell Immunol* 2003; **223**: 1.
37. Sordi V, Bianchi G, Buracchi C, *et al.* Differential effects of immunosuppressive drugs on chemokine receptor CCR7 in human monocyte-derived dendritic cells: selective upregulation by rapamycin. *Transplantation* 2006; **82**: 826.
38. Jones DL, Sacks SH, Wong W. Controlling the generation and function of human CD8+ memory T cells in vitro with immunosuppressants. *Transplantation* 2006; **82**: 1352.
39. Mira E, Leon B, Barber DF, *et al.* Statins induce regulatory T cell recruitment via a CCL1 dependent pathway. *J Immunol* 2008; **181**: 3524.
40. Zeiser R, Nguyen VH, Beilhack A, *et al.* Inhibition of CD4+CD25+ regulatory T-cell function by calcineurin-dependent interleukin-2 production. *Blood* 2006; **108**: 390.
41. Coenen JJ, Koenen HJ, van Rijssen E, *et al.* Rapamycin, not cyclosporine, permits thymic generation and peripheral preservation of CD4+ CD25+ FoxP3+ T cells. *Bone Marrow Transplant* 2007; **39**: 537.
42. Baan CC, van der Mast BJ, Klepper M, *et al.* Differential effect of calcineurin inhibitors, anti-CD25 antibodies and rapamycin on the induction of FOXP3 in human T cells. *Transplantation* 2005; **80**: 110.
43. D'Cruz LM, Klein L. Development and function of agonist-induced CD25+Foxp3+ regulatory T cells in the absence of interleukin 2 signaling. *Nat Immunol* 2005; **6**: 1152.
44. Campbell DJ, Ziegler SF. FOXP3 modifies the phenotypic and functional properties of regulatory T cells. *Nat Rev Immunol* 2007; **7**: 305.

