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ORIGINAL ARTICLE

Increased frequency of regulatory T Cells and selection of highly potent CD62L+ cells during treatment of human lung transplant recipients with rapamycin

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Summary

The currently available immunosuppressive agents applied in human transplantation medicine are highly potent in the protection from acute allograft rejection. However, long-term allograft survival is still poor as these drugs fail to sufficiently prevent chronic allograft rejection. Naturally occurring regulatory T cells have been postulated as the key players to establish long-lasting transplantation tolerance. Thus, the development of immunosuppressive regimens which shift the pathological balance of cytopathic versus regulatory T cells of human allograft recipients towards a protective T-cell composition is a promising approach to overcome limitations of current transplantation medicine. Thirty-three patients that received rapamycin (RPM) or calcineurin inhibitor treatment following lung transplantation were included and their T-cell compartments analysed. Twelve healthy volunteers without history of lung disease served as controls. In this article, we show that treatment of human lung transplant recipients with RPM is associated with an increased frequency of regulatory T cells, as compared with treatment with calcineurin inhibitors or to healthy controls. Moreover, regulatory T cells during treatment with RPM were CD62Lhigh, a phenotype that displayed an enhanced immunosuppressive capacity *ex vivo*. Our data support the use of RPM in human lung transplant recipients and undertaking of further prospective studies evaluating its impact on allograft and patient survival.

Introduction

In the last decades, highly potent immunosuppressive agents have been developed, which enabled substantial progress in human organ transplantation. Cyclosporin A (CsA) and tacrolimus (FK506) are potent inhibitors of calcineurin, which under normal circumstances induces the transcription of interleukin-2 (IL-2). Unlike the calcineurin inhibitors, that affect the first phase of the T-lymphocyte activation, rapamycin (RPM, syn. sirolimus) affects the second phase, namely the signal transduction and clonal proliferation of T lymphocytes. The reduction of lymphokine production and interleukin release by above immunosuppressant agents lead to reduced function of effector T cells (Teff), which in turn prevents acute allograft rejection and markedly improves rates of short-term allograft survival [1]. However, long-term application of the currently recommended immunosuppressive regimens is burdened with severe side-effects and their effect on chronic allograft rejection is poor; both reasons are responsible for the disappointing long-term results after allograft transplantation [2]. Human lung transplant recipients are in particular affected by these issues, because the high immunogenicity of the lung

requires a comparably aggressive immunosuppression [2]. In view of these issues, there is strong need for improved immunosuppressive strategies that can establish specific transplantation tolerance, without continual general immunosuppression.

The development of immunosuppressive regimens which shift the pathological balance of cytopathic versus regulatory T cells (Tregs) of human allograft recipients towards a protective T-cell composition is a promising approach to overcome limitations of current transplantation medicine. Recent studies in animal models have confirmed that CD4+CD25+Foxp3+ Tregs are crucial to sustain ongoing transplant tolerance [3,4]. For example, it was shown that the transfer of Tregs alone without any additional immunosuppressive agents is sufficient to avoid solid allograft rejection in animal models [3]. Initially, Tregs were thought to exclusively develop in the thymus through positive selection. However, more recent studies suggest that Tregs can also develop in peripheral compartments from naïve CD4+ T cells in an antigen-specific manner, mediated by peptide-presenting dendritic cells [4–7]. This is an important prerequisite for the development of allograft-specific Tregs, as the thymus does not contain any allo-antigen. Studies in animal models and *in vitro* revealed that different immunosuppressive drugs can promote or abrogate *de novo* generation of Tregs. For example, RPM was shown to support the *de novo* generation of Tregs in the periphery of mice after skin transplantation [8]. In contrast, application of CsA resulted in a strong reduction of the peripheral Treg compartment in mice.

As up to this point, the role of Tregs in human allograft recipients still needs to be better characterized. The frequency of Tregs in the circulation and in biopsy specimens of renal and lung allograft recipients has been correlated positively with allograft survival [9–13]. Accordingly, acute liver rejection was shown to be associated with decreased numbers of Tregs [14]. However, as studies evaluating Tregs and Teffs in human allograft recipients under different immunosuppressive regimens are rare, more studies are necessary to translate these convincing experimental data into clinical practice.

In this study, we aimed to characterize the effect of currently recommended immunosuppressive regimes on the frequency and function of Tregs in human lung transplant recipients. In this study, we show for the first time that treatment of human lung transplant recipients with RPM in combination with mycophenolate mofetil (MMF) is associated with an increased frequency of Tregs, as compared with treatment with calcineurin inhibitors or to healthy control individuals. Moreover, during treatment with RPM/MMF, Tregs were CD62Lhigh, a phenotype that displayed an enhanced immunosuppressive capacity *ex vivo*. In contrast, alterations in the phenotype of Teff during immunosuppression, namely a shift towards Th2 cell predominance (Pred), were independent of the applied compounds.

Materials and methods

Patients and clinical samples

Thirty-three lung transplant recipients (mean age: 50.6 years, range 21–74 years) were selected from lung transplants performed at the University Hospital Frankfurt, Germany, between 1986 and 2006 according to the following inclusion criteria: post-transplant follow up for at least 12 months, stable clinical condition without any signs of infection or acute allograft rejection at time of enrolment, treatment with the same immunosuppressive regimen for at least 4 months prior to enrolment. Patients were divided into three groups according to their immunosuppressive regimen (CsA, $n = 14$; FK506, $n = 12$; RPM, $n = 7$). All patients additionally received MMF and low-dose Pred (5–10 mg/day p.o.). Plasma concentrations of immunosuppressive drugs were: cyclosporine 120–180 ng/ml, tacrolimus 6–8 ng/ml, rapamycin 6–8 ng/ml and MMF 1.5–3 ng/ml. Treatment indication for rapamycin was a reduction of nephrotoxicity during immunosuppressive therapy in all seven patients. Twelve healthy volunteers without a previous history of lung disease served as controls. Demographic and clinical data are summarized in Table 1. Peripheral ethylene diamine tetraacetic acid (EDTA)-blood was taken from all patients and healthy controls by peripheral vein puncture. Institutional board approval and informed consent from all patients was obtained in accordance with the Helsinki declaration.

Reagents and antibodies

For flow cytometry, the following antihuman monoclonal antibodies were used (all fluorochrome-conjugated): anti-CD4-fluorescein isothiocyanate (FITC), anti-CD4-PercP, anti-CD4-APC, anti-CD25-phycoerythrin (PE), anti-CD25-FITC, anti-CD62L-FITC, anti-CD127-PE, anti-ICOS-PE, anti-BTLA-PE (all from Pharmingen, Heidelberg, Germany), anti-Foxp3-PE, anti-Foxp3-APC, anti-IFN-gamma-PE, anti-IL-4-PE (all from Miltenyi Biotec, Bergisch Gladbach, Germany) and anti-Tim3-PE (RD Systems, Wiesbaden, Germany). Appropriate immunoglobulin isotypes were used as a control. Cytofix/Cytoperm and Wash/Perm for intracellular cytokine staining were from BD Biosciences (Heidelberg, Germany). CFSE was obtained from Invitrogen (Karlsruhe, Germany). Magnetic cell separation was performed with commercially available kits for isolation of CD4+CD25+ Tregs, CD4+ T cells, naïve CD4+ T cells, and CD62L T cells (all from Miltenyi Biotec).

Table 1. Demographic and baseline characteristics of study population.

	Cyclosporine	Tacrolimus	Rapamycin	Control	*P*-value
Gender, *n* (%)					
Female	8	7	5	6	NS
Male	6	5	2	6	
Age, years					
Median	51	64	55	46	NS
Range	21–72	23–74	29–67	22–65	
BMI, kg/m^2					
Median	20.9	22.9	20.7	20.7	NS
Range	15.3–28.6	15.2–32.8	15.0–34.0	16.3–27.8	
CRP, mg/dl					
Median	0.1	0.1	0.2	ND	NS
Range	0.02–1.08	0.07–1.82	0.09–1.2		
Blood leukocytes, per nl					
Median	7.14	7.72	10.0	ND	NS
Range	3.0–11.3	4.5–13.0	5–13.7		
Underlying disease					
CF	7	4	3		NS
COPD	5	7	4		
UIP	2	1	0		
Years after LTX					
Median	4	6	7		NS
Range	1.0–20	2.0–12	2.0–8		

BMI, body mass index; CRP, C-reactive protein; CF, cystic fibrosis; COPD, chronic obstructive pulmonary disease; UIP, ususal interstitial pneumonitis; LTX, lung transplantation; NS, not significant; ND, not determined.

Plasma concentrations of immunosuppressive drugs were: cyclosporine 120–180 ng/ml, tacrolimus 6–8 ng/ml, rapamycin 6–8 ng/ml and MMF 1.5–3 ng/ml.

P-value indicates statistical significance between immunosuppressive treatment arms.

For *in vitro* T-cell stimulation, a T-cell activation/expansion kit was used, which is based on beads coated with anti-CD3 and anti-CD28 from Miltenyi Biotec. For *in vitro* experiments the following immunosuppressive agents were used: Cyclosporine A (CsA; Novartis Pharma, Basel, Switzerland), tacrolimus (FK506; Astellas, Munchen, Germany) and rapamycin (RPM; Wyeth Laboratories, Philadelphia, PA, USA).Except where indicated, all other chemicals were reagent-grade and purchased from Sigma Aldrich (St. Louis, MO, USA).

Fluorescence staining and flow cytometry

Lymphocytes from freshly drawn peripheral EDTA-blood were isolated by Histopaque gradient and used immediately. To determine the absolute cell numbers of the indicated lymphocyte populations the totality of all live lymphocytes was counted first by using the trypan blue method. By using these total cell numbers of all lymphocytes the absolute cell numbers of the indicated subpopulations were calculated based on their percentages on all events during flow cytometric analysis. Fc-receptor blocking was performed with human IgG (Sigma Aldrich). For staining of extracellular antigens, approx. 10^5 cells were incubated for 20 min with the indicated antibody at room temperature in the dark, washed twice with PBS containing 1% bovine serum albumin and re-suspended in 20 µl buffer. For staining of intracellular antigens, approx. 10^5 cells were first stained for extracellular antigens, followed by fixation and permeabilization with Cytofix/Cytoperm for 20 min at room temperature. Then, cells were washed twice with Wash/Perm and incubated with anti-cytokine antibodies or anti-FoxP3 antibodies in 20 µl Wash/Perm for 30 min at room temperature. All antibodies were titrated for optimal concentrations. Then cells were washed twice, resolved in 50 µl PBS and analysed by flow cytometry. Fluorescence-activated cell sorting was performed on live cells with a FacsCalibur System (Becton Dickinson, Heidelberg, Germany) and analysed with Summit software (v4.1; Dakocytomation, Freiburg, Germany).

T-cell stimulation and *ex vivo* exposure to immunosuppressive agents

For *ex vivo* experiments, naïve CD4+ T cells were isolated from freshly drawn peripheral EDTA-blood of healthy volunteers ($n = 3$ each) by magnetic cell separation (MACS)-technology, according to the manufacturer's

instructions (Miltenyi Biotec). Then 10^4 CD4+ T cells were stimulated by using the above described MACS T-cell activation/expansion kit following the manufacturer's recommendations. Briefly, cells were cultured in 96-well plates in 200 µl Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum in an atmosphere containing 5% CO_2. Concentrations of anti-CD3/CD28-beads were chosen and titrated according to the manufacturer's instructions. Immunosuppressive agents were added at day 0 at the following concentrations (final) [15]: CsA, 100 ng/ml; FK506, 10 ng/ml; RPM, 10 and 100 ng/ml (as indicated). Following incubation cells were washed twice and processed for flow cytometric analysis of Foxp3, CD25 and CD62L as described above. Other aliquots were analysed for cell proliferation and apoptosis as described below.

Treg suppression assay

Suppressive function of Tregs was assessed by using an *in vitro* suppression assay. Direct *ex vivo* isolation of the indicated T-cell populations was performed by using MACS-isolation kits from Miltenyi Biotec according to the manufacturer's instructions. Freshly isolated CD4+CD25− T cells (responders) from the blood of lung transplant recipients and healthy donors ($n = 3$ each) were stained with CFSE for 20 min and then washed four times. Anti-CD3/anti-CD28 coated beads were used as a stimulus (Miltenyi Biotec). Cell culture was performed in 96-well plates in 200 µl IMDM supplemented with 10% fetal calf serum in an atmosphere containing 5% CO_2. CD4+CD25− T cells (10^4) were stimulated for 2 h. Next, the indicated numbers of CD4+CD25+CD62L− or CD4+CD25+CD62L+ T cells were added. To separate CD4+CD25+CD62L+ cells from CD4+CD25+CD62L− cells, washed lymphocytes were selected or depleted with regard to their expression of CD62L by using CD62L MACS-isolation kit and subsequently purified by using the CD4+CD25+ Treg MACS-isolation kit. After 72 h, the percentage of proliferating responder cells as reflected by CFSE dilution was assessed by flow cytometry.

Apoptosis assay

Lymphocytes were harvested as described above and apoptosis was quantified by Annexin V staining using a kit (Miltenyi Biotec). Briefly, lymphocytes (10^6) were washed twice with Annexin V binding buffer, resuspended in 100 µl binding buffer and stained with 10 µl Annexin V according to the manufacturer's instructions. Then, cells were incubated for 15 min at room temperature, washed again twice and analysed immediately by flow cytometry.

Statistics

Data are presented in text and tables in terms of frequencies and means with standard deviation and range, as appropriate. Differences between subgroups were statistically compared by Student's *t*-test. The general level for assessment of statistical significance was predefined as $2a = 0.05$ (two-sided). Resulting *P*-values were presented descriptively without further adjustment for multiple testing. All statistical calculations were performed using statistical Analysis (SAS) software, version 9.1 (SAS Institute Inc., Cary, NC, USA).

Results

Number, phenotype and cytokine production of effector CD4+ T cells do not differ among different immunosuppressive regimes

The influence of the different immunosuppressive regimens on the size of potentially allo-protective and allo-destructive CD4+ T cell compartments in the peripheral blood of human lung transplant recipients was quantified by flow cytometry. Absolute numbers of lymphocytes were substantially decreased in human lung transplant recipients (mean 1.5–1.9/nl, $P < 0.05$), compared to healthy control individuals (4.3/nl) (Fig. 1a). Similar findings were made when the subgroup of CD4+ T cells was analysed (0.39–0.57/nl vs. controls 1.7/nl, respectively, $P < 0.05$) (Fig. 1b). However, there was no difference for both lymphocyte and CD4+ T-cell counts among the immunosuppressive treatment arms ($P > 0.05$).

To assess whether different immunosuppressive treatment regimes are associated with phenotypic alterations of circulating Teff, expression of the signature cytokines IFN-γ and IL-4, as well as of negative and positive co-stimulatory molecules was determined in CD4+ T cells by flow cytometry. The expression of IFN-γ was reduced in human lung transplant recipients in all treatment arms when compared to the control group (10.4–12.7% vs. 18.0% respectively) (Fig. 1c). In contrast, the expression of IL-4 was markedly increased in all patient groups when compared to the control group (16.5–22.1% vs. 9.2%, respectively, $P < 0.05$) (Fig. 1d). Thus, in human lung transplant recipients the proportions of Th1 and Th2 cells are changed towards a Th2-phenotype during immunosuppressive therapy, independently of the applied agents.

The expression of the positive co-stimulatory molecule *Tim3* was significantly reduced in allograft recipients compared to the control group (9.7–15.1% vs. 23.8% respectively) (Fig. 2a). Comparable results were obtained for the expression of the inducible T-cell co-stimulator *ICOS* (allograft recipients 4.92–6.25% vs. controls 13.5%,

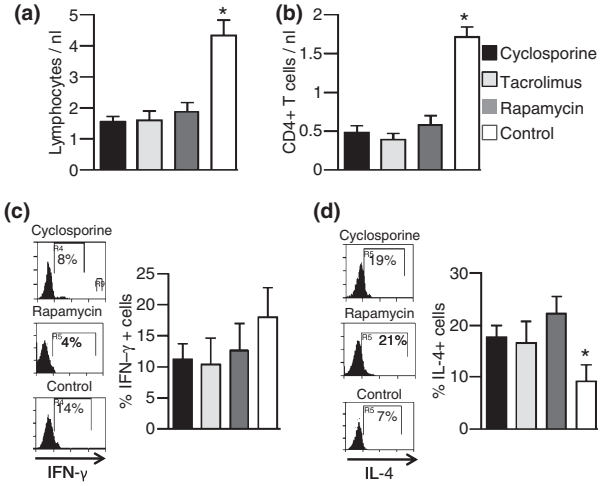

Figure 1 Immunosuppressive therapy induces a Th2-dominance in human lung transplant recipients. (a) Indicated immunosuppressive therapies (+MMF/Pred) substantially decrease total numbers of lymphocytes in peripheral blood of lung transplant recipients. (b) Similar findings were made when the subgroup of CD4+ T cells was analysed. Differences among immunosuppressive agents were not statistically significant. Decreased expression of IFN-γ (c) and increased expression of IL-4 (d) in peripheral blood CD4+ T cells under immunosuppressive therapy. The data demonstrate that all indicated immunosuppressive agents induce a shift towards a Th2-dominance. Exemplary histograms of the indicated cytokines are shown for cyclosporine- and rapamycin-treated patients and healthy individuals (control). Bars indicate mean ± SEM (*$P < 0.05$ for differences between controls versus patients).

respectively, $P < 0.05$) (Fig. 2b). In contrast, the inhibitory co-stimulator *BTLA* was strongly expressed on peripheral CD4+ T cells during immunosuppressive therapy compared to the control group (76.8–81.1% vs. 50.9%, respectively, $P < 0.05$) (Fig. 2c). Of note, the expression of the positive (*Tim3, ICOS*) and the inhibitory (*BTLA*) co-stimulants between immunosuppressive regimens was comparable ($P > 0.05$). Thus, Teff predominantly express negative co-stimulatory molecules during immunosuppression with both calcineurin inhibitor- and RPM-based combination therapies.

Increased numbers of regulatory T cells following RPM therapy

To investigate the impact of the different immunosuppressant regimes on specific immunomodulatory function of lymphocytes, we analysed the frequency of Tregs. The absolute number of CD4+CD25+Foxp3+-Tregs in lung transplant recipients was significantly higher following treatment with RPM/MMF/Pred (0.10/nl), when compared to calcineurin inhibitor-based therapy with CsA/MMF/Pred or FK506/MMF/Pred (0.05/nl, $P < 0.05$, each)

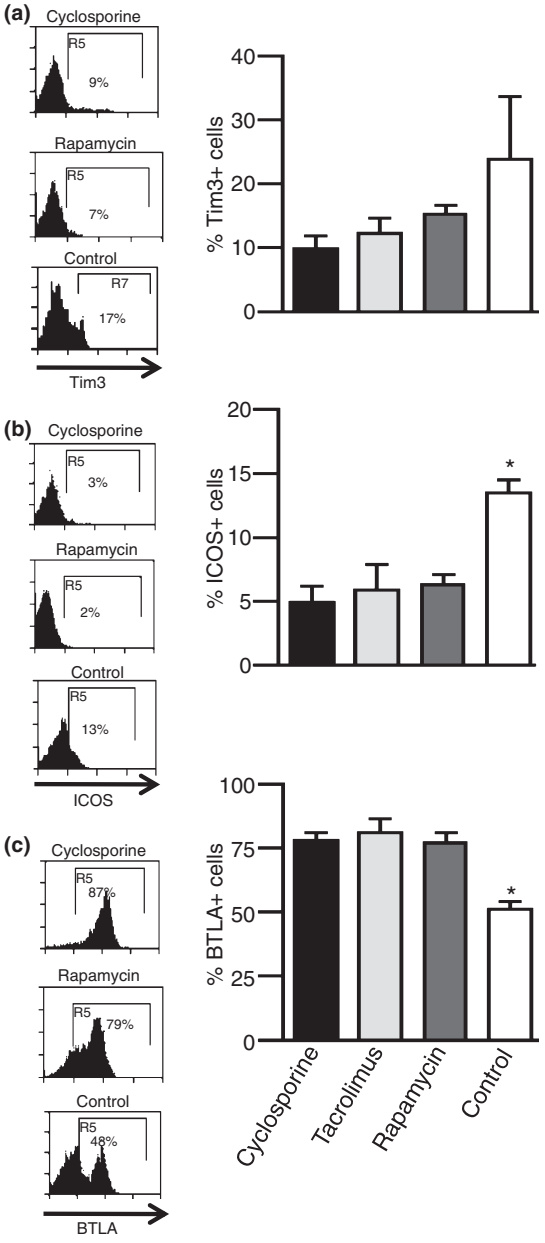

Figure 2 Immunosuppressive therapy alters the phenotype of effector T cells in human lung transplant recipients. (a,b) Indicated immunosuppressive therapies (+MMF/Pred) down-regulate the expression of positive co-stimulatory molecules *Tim3* and *ICOS* on effector CD4+ T cells of lung transplant recipients. (c) In contrast, the inhibitory co-stimulator BTLA is up-regulated. No significant differences in phenotype profile were detected among patients treated with different immunosuppressive regimens. Exemplary histograms of the indicated effector molecules are shown for cyclosporine- and rapamycin-treated patients and healthy individuals (control). Bars indicate mean ± SEM (*$P < 0.05$ for differences between controls versus patients).

(Fig. 3a). Of note, the absolute numbers of Tregs under RPM-based therapy was comparable to healthy controls individuals (0.10/nl). The mean relative number of Tregs

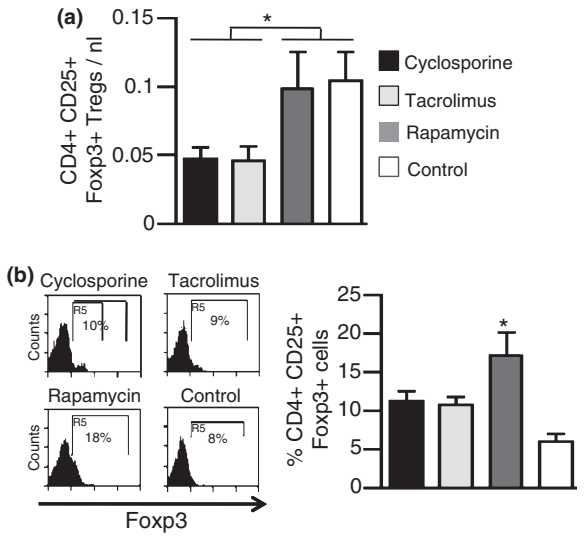

Figure 3 Increased numbers of regulatory T cells following RPM therapy of human lung transplant recipients. Mean absolute numbers of CD4+CD25+Foxp3+ regulatory T cells (a) and relative numbers of CD4+CD25+Foxp3+ regulatory T cells of all CD4+ T cells (b) in the peripheral blood of human lung transplant recipients under the indicated immunosuppressive therapies (+MMF/Pred). During treatment with RPM, absolute and relative numbers of CD4+CD25+Foxp3+ regulatory T cells are higher as compared with calcineurin inhibitor-based therapies and healthy individuals (control). Exemplary histograms of the CD4+CD25+Foxp3+ cells are shown on the left. Bars indicate mean ± SEM (*$P < 0.05$ for differences between RPM versus CsA/FK506 and controls versus CsA/FK506).

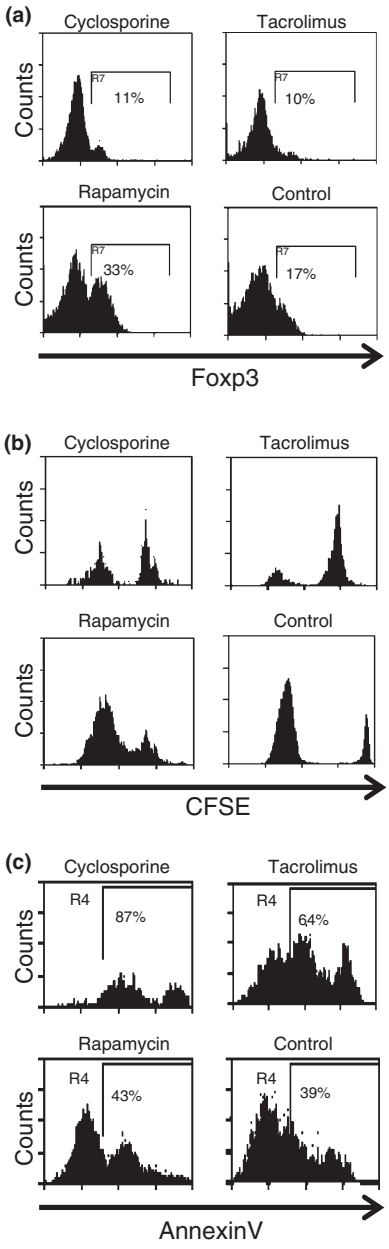

Figure 4 Foxp3-expression, cell proliferation and apoptosis of CD4+ T cells in the presence of different immunosuppressive agents *ex vivo*. Naïve CD4+ T cells were isolated from peripheral blood of healthy volunteers by MACS-sorting and then stimulated with anti-CD3 and anti-CD28 in the presence of the indicated immunosuppressive agents or PBS (control). After 3 days of culture, Foxp3-expression (a), cell proliferation (CFSE intensity) (b), and apoptosis (percentage of AnnexinV positive cells) (c) were determined by flow cytometry. Representative histograms show that the proportion of Foxp3-expression and rates of cell proliferation are significantly higher, but the percentage of apoptotic cells was lower in the presence of RPM as compared with calcineurin inhibitors.

on all CD4+ T cells for the RPM regime (17.2%) was significantly higher when compared to both calcineurin-inhibitors (CsA, 10.8; FK506, 11.2%, $P < 0.05$), as well as to the control group (6.0%, $P < 0.05$) (Fig. 3b). Thus, absolute, but not relative numbers of Tregs are decreased during combination therapy with calcineurin inhibitors. In contrast, both absolute and relative numbers of Tregs are markedly enhanced in human lung transplant recipients during treatment with combination therapy with RPM, as compared with treatment with calcineurin inhibitor-based therapies. Interestingly, RPM-based therapy results in increased relative numbers of Tregs as compared with the healthy control individuals. Of note, intra-individual Treg counts remained stable over time, as determined by sequential FACS analysis over 5 months in a subset of patients ($n = 6$, data not shown).

Induction of Foxp3-expression by RPM *ex vivo*

To examine the impact of immunosuppressive agents on differentiation of Tregs, we analysed Foxp3 expression in naïve polyclonal CD4+ T cells following *ex vivo* activation with anti-CD3 and anti-CD28. Activated CD4+ T cells

expressed markedly higher levels of Foxp3 after 3 days of culture in the presence of RPM (28.1%) compared to calcineurin inhibitors (CsA, 10.3; FK506, 13.4%) or PBS (10–20%) (Fig. 4a). Equivalent to Foxp3 the majority of these CD4+Foxp3+ T cells up-regulated CD25 (data not shown). In parallel, cell proliferation was higher in the presence of PRM than in the presence of calcineurin inhibitors, as assessed by carboxyfluorescein diacetate succinimidyl ester (CFSE)-staining (Fig. 4b). To assess whether rates of apoptotic cells differ in stimulated CD4+ T cells as a function of the different immunosuppressive agents used, CD4+ T cells were stained with AnnexinV after 3 days of cell culture. The percentage of AnnexinV positive CD4+ T cells was significantly lower when cultured in the presence of RPM (30–45%) compared to calcineurin inhibitors (CsA, 70–90%; FK506, 60–80%) (Fig. 4c). Of note, the percentage of apoptotic cells following culture with RPM or placebo (30–50%) was comparable ($P > 0.05$).

Regulatory T cells during RPM-based immunosuppression are CD62Lhigh

Based on the expression of surface receptors, CD4+ Teff can be further divided into functionally distinct populations. In some animal models, CD4+CD25+CD62L+ Tregs have been shown to serve as essential suppressors of autoimmunity [5]. To assess the impact of differential immunosuppressive therapy on CD62L+ receptor expression, we quantified CD62L+ concentration on CD4+CD25+Foxp3+-Tregs by flow cytometry. Following treatment of patients with RPM/MMF/Pred, large fractions of Tregs were found positive for CD62+ (37.7%). In contrast, the fractions of Tregs that were positive for CD62+ were significantly lower following treatment of patients with either CsA/MMF/Pred (23.2%), FK506/MMF/Pred (20.4%) or in healthy control individuals (17.6%), $P < 0.05$ each (Fig. 5). Thus, RPM-based immunosuppressive therapy is associated with an accumulation of CD62L+ Tregs in peripheral blood of human lung transplant recipients.

CD62Lhigh regulatory T cells have increased immunosuppressive capacity *ex vivo*

Earlier studies in some animal models have shown enhanced immunosuppressive capacity of Tregs following co-incubation with RPM *ex vivo* [16]. In the light of our above findings, we postulate that enhanced immunosuppressive capacity following RPM treatment is mediated by an extended compartment of CD62L+ cells. To investigate the impact of CD62L+ Tregs on immunosuppression, we isolated CD4+CD25+CD62L+ and CD4+CD25+CD62L− T cells by MACS from peripheral blood of healthy individuals and human lung transplant recipients treated with RPM-based regimen. The obtained cell populations were at least 80% Foxp3-positive, as quantified by flow cytometry. Next, the immunosuppressive capacity of CD4+CD25+CD62L+ and CD4+CD25+CD62L− T cells was examined by incubation with polyclonal, stimulated, CFSE-labelled, naïve CD4+CD25− T cells for 72 h. Under our experimental conditions, the capacity for suppression of activated Teff was found significantly higher following incubation with CD62L+ Tregs as compared with CD62L− Tregs ($P < 0.05$) (Fig. 6a and b). Of note, the immunosuppressive capacity of CD62L+ Tregs from RPM-treated lung transplant recipients and healthy individuals was equivalent (Fig. 6b and c) ($P > 0.05$). Thus, RPM-based immunosuppressive therapy is associated with the presence of a high proportion of CD62Lhigh Tregs, which display enhanced immunosuppressive qualities.

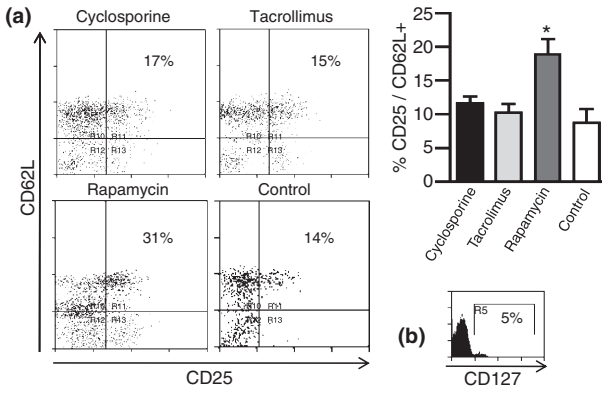

Figure 5 Regulatory T cells are CD62Lhigh in human lung transplant recipients treated with RPM. (a) Representative scatter plots (left) show co-expression of CD25 and CD62L in CD4+Foxp3+ Tregs that were isolated from the peripheral blood of human lung transplant recipients under the indicated immunosuppressive therapies (+MMF/Pred). As compared with calcineurin inhibitor-treated patients and to healthy individuals (controls), RPM-based immunosuppressive therapy is associated with increased numbers of CD62Lhigh Tregs. Bars indicate mean ± SEM (*$P < 0.05$ for differences between RPM versus CsA, FK506 and controls). (b) A representative histogram of CD127-expression on CD4+CD25+CD62L+ T cells is shown. CD4+CD25+CD62L+ T cells express low levels of CD127.

Discussion

In this study, we characterized for the first time the Teff and Treg compartment in the peripheral blood of human lung transplant recipients receiving combination therapies of CsA/MMF/Pred, FK506/MMF/Pred and RPM/MMF/Pred. In this study, we show that the frequency of Teff is substantially reduced in all groups as compared with

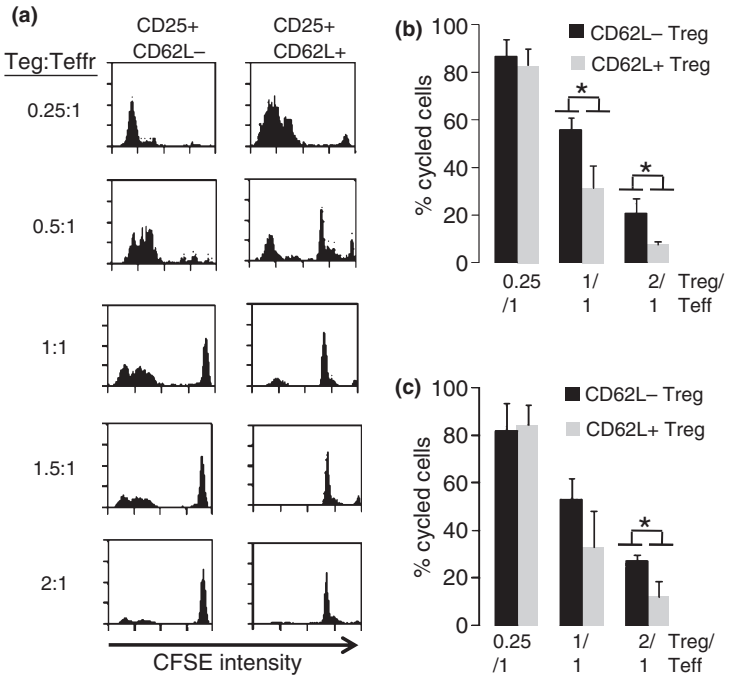

Figure 6 CD62L high regulatory T cells show enhanced immunosuppressive capacity *ex vivo*. (a) Freshly isolated CD4+CD25– T cells from the blood of healthy donors were stained with CFSE and stimulated with anti-CD3 and anti-CD28. The indicated ratios of CD62L–:CD62L+ Tregs were added and CFSE-intensity was determined by flow cytometry after 72 h. The capacity for suppression of activated effector T cells was significantly higher following incubation with CD62L+ as compared with CD62L– Tregs. Exemplary histograms of the CFSE intensity are shown. (b) Results of three separate experiments as described in (a) are shown. Results are expressed as the percentage of cycled CFSE+ cells as: (number of cycled CFSE+ cells/total number of cycled and noncycled cells). (c) Results of three experiments as described in (a) and (b) are shown with the difference that regulatory and effector T cells were obtained from human lung transplant recipients who were treated with rapamycin. There are no significant differences in immunosuppressive capacity of Tregs from healthy control individuals or human lung transplant recipients ($P > 0.05$). Bars indicate mean ± SEM (*$P < 0.05$ for differences between suppressive capacity of CD62L– and CD62L+ Tregs).

healthy individuals. In contrast, the Treg compartment is exclusively enlarged in individuals exposed to RPM-based treatment. Absolute numbers of Tregs under RPM treatment were even slightly higher than in healthy individuals. In contrast, CsA- or FK506-based treatment resulted in marked reduction of the Tregs compartment.

Our data confirm and extend the findings of previous studies that postulated a differential effect between the above-mentioned immunosuppressive agents on graft-destructive Teff and graft-protective Tregs. It was shown that CsA inhibits, but RPM promotes activation-induced cell death of Teff [17]. Several studies revealed detrimental effects of calcineurin inhibitors such as CsA or FK506 on the survival and function of Tregs [18,19], which can be explained by the calcineurin inhibitor-induced interruption of IL-2-signalling, a key cytokine in Treg development and homeostasis [19]. In contrast, RPM-exposure can promote *de novo* generation and gain of Treg function *in vitro* [8,18,20,21]. However, as studies evaluating Tregs and Teff in human allograft recipients under different immunosuppressive regimens are rare, these convincing experimental data have not been translated into clinical practice so far.

Because all patients in this study received combination therapies comprising MMF and low-dose Pred, our setting does not unarguably allow attributing the observed effects to RPM. However, previous *in vitro* studies did not find a significant effect of MMF alone on the Treg compartment [19]. Pred was shown to promote Treg survival *in vitro* and *in vivo* [22]. In this study, co-administration of MMF and Pred was similar in all groups and the dosage of Pred was low in all patients. Thus, RPM seems to play the pivotal role in the enlargement of the Treg compartment. As combination regimens are still mandatory in human lung transplant recipients, characterization of Tregs during such therapies is of considerable interest and thus reflects clinical scenarios.

Previous studies in mice have postulated a particular role of CD62Lhigh Tregs. For example, CD62Lhigh, but not CD62Llow Tregs were able to inhibit diabetes development in nonobese diabetic mice [5]. Importantly, these CD62Lhigh Tregs could be selectively expanded *in vitro*

by dendritic cells without losing their phenotype [5]. In addition, it was shown that CD62Lhigh but not CD62Llow Tregs are highly potent in suppressing acute graft-versus-host disease in mice [23].In this study, we found that the subset of CD62Lhigh Tregs is increased in human lung transplant recipients during RPM-based treatment as compared with individuals treated with calcineurin inhibitors and also as compared with healthy control individuals. Moreover, we observed a superior immunosuppressive capacity of CD62Lhigh Tregs *ex vivo*, as compared with CD62Llow Tregs. These data are the first, which characterize CD62Lhigh Tregs in human allograft recipients and indicate a favourable immune-modulating effect of RPM. Together with the quantitatively enlarged Treg compartment, the selection of CD62Lhigh Tregs during RPM-based immunosuppression is supportive of a significantly enhanced immunosuppressive capacity of the Treg pool. As CD62L is an integrin enabling recirculation through lymphoid tissues, the selection of CD62Lhigh Tregs might be an essential feature of host immunity. CD62L expression characterizes memory Teff and Treg populations, which are determined to home to the lymph nodes in contrast to inflammation-seeking CD62Llow T cells [24–26]. Induction of CD62Lhigh Tregs under RPM-based treatment might inhibit the priming of naïve allograft-specific T cells in the draining lymph nodes and thus help to prevent chronic allograft rejection.

In theory, there are at least two ways for expansion of the Treg compartment in human allograft recipients: First, their generation or gain of function could be induced *in vivo* by specific compounds. Second, Tregs could be *de novo* generated *in vitro* and transferred to the recipient thereafter. *In vivo*, the extrathymic *de novo* generation of Tregs is pivotal, because mainly those Tregs are allo-antigen specific [27]. Whether a naïve T cell differentiates into a Treg or a Teff seems to crucially depend on the local cytokine milieu. *In vitro* experiments have shown that RPM inhibits the production of pro-inflammatory cytokines and promotes the expression of TGF-beta [15,28]. Together with its inhibitory impact on the cell cycle, RPM might use this pathway to induce the *de novo* generation of Treg. Thus, it can be speculated that the high number of Tregs during RPM-based therapy is at least partially attributable to peripheral *de novo* generation from naïve T cells. In this study, we show that activation of naive CD4+ T cells in the presence of rapamycin but not of calcineurin inhibitors results in the induction of Foxp3. This finding might reflect a differentiation of naive T cells to Tregs in the presence of rapamycin *in vitro*. However, this cannot be established definitely with our experiments as a recent study has shown that Foxp3 can be expressed transiently in Teff as well [29].

In our study, we did not observe differences in the Teff compartment during treatment with rapamycin as compared with calcineurin inhibitors. In all treatment arms, we report a polarization towards a Th2 cell phenotype, which is indicated by a reduced expression of IFN-γ and Tim3 on the one hand and an enhanced expression of IL-4 and BTLA on the other hand. Tim3, which is predominantly expressed on Th1 cells, seems to play an important role in the maintenance of immune tolerance as engagement of Tim3 by its receptor galectin 9 results in apoptosis of Tim3-expressing T cells [30]. The functional consequences of low ICOS expression, which we have observed in all human lung transplant recipients, are less clear. ICOS plays a complex role in adaptive immunity, which is indicated by the observation that loss of ICOS can result in common variable immunodeficiency [31]. In addition, it was shown that Tregs within pancreatic islets of diabetic mice critically depend on ICOS-signalling [32]. Low ICOS-expression during immunosuppressive therapy might be explained by the fact that ICOS-expression depends on T-cell activation, which is abrogated in the presence of immunosuppressive agents. Because recent data indicate a role of Th17 cells in allograft rejection, investigating these cells in patients during treatment with rapamycin is of interest for further studies [33].

In conclusion, we show that the frequency of highly immunosuppressive CD62Lhigh Tregs in the peripheral blood of human lung transplant recipients is significantly increased during RPM-based combination therapy, as compared with calcineurin inhibitor-based treatment and to healthy control individuals. Thus, our data suggest that RPM can be used to preferentially promote Treg-mediated transplantation tolerance. Prospective studies in human allograft recipients evaluating potential benefits of RPM-based therapy on patient and allograft survival are required to determine the clinical significance of these findings.

Authorship

CML, SJ, TOW and TOH: planning the study. CML, TYT and HF: collecting the data. CML, TYT and TOH: analysis of data. CML, TYT, HF, SJ, TB, TOW and TOH: preparation and revision of the manuscript.

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