Rapamycin, and not cyclosporin A, preserves the highly suppressive $CD27^+$ subset of human $CD4^+CD25^+$ regulatory T cells

Jeroen J. A. Coenen, Hans J. P. M. Koenen, Esther van Rijssen, Luuk B. Hilbrands, and Irma Joosten

The immunosuppressive drugs rapamycin and cyclosporin A (CsA) are widely used to prevent allograft rejection. Moreover, they were shown to be instrumental in experimental models of tolerance induction. However, it remains to be elucidated whether these drugs have an effect on the $CD4^+CD25^+$ regulatory T-cell (T_{REG}) population, which plays an important role in allograft tolerance. Recently, we reported that alloantigen-driven expansion of human $CD4^+CD25^+$ T_{REG}s gives rise to a distinct highly suppressive $CD27^+T_{REG}$ subset next to a moderately suppressive $CD27^-T_{REG}$ subset. In the current study we found that rapamycin and CsA do not interfere with the suppressive activity of human naturally occurring $CD4^+CD25^+$ T cells. However, in contrast to CsA, rapamycin preserved the dominance of the potent $CD27^+T_{REG}$ subset over the $CD27^-T_{REG}$ subset after alloantigen-driven expansion of $CD4^+CD25^+$ T_{REG}s in vitro. Accordingly, $CD4^+CD25^+$ T_{REG}s cultured in the presence of rapamycin displayed much stronger suppressive capacity than $CD4^+CD25^+$ T_{REG}s cultured in the presence of CsA. In addition, $CD4^+CD25^+$ T_{REG} cells cultured in the presence of rapamycin, but not CsA, were able to suppress ongoing alloimmune responses. This differential effect of rapamycin and CsA on the $CD27^+T_{REG}$ subset dominance may favor the use of rapamycin in tolerance-inducing strategies. (Blood. 2006; 107:1018-1023)

Introduction

The development of an alloimmune response into rejection or stable allograft tolerance is strongly determined by the balance between alloreactive effector cells and $CD4^+CD25^+$ regulatory T cells (T_{REG}s).[1,2] Currently used immunosuppressive drugs are efficient in preventing allograft rejection by reducing effector T-cell expansion. However, it remains to be elucidated whether these drugs antagonize the induction of tolerance by affecting the naturally occurring T_{REG} population.

$CD4^+CD25^+$ T_{REG}s exert their immunosuppressive effect after activation by T-cell receptor (TCR) triggering.[3] Moreover, it has recently been demonstrated that signaling through the interleukin-2 (IL-2) receptor is crucial for the functional activity of T_{REG}s.[4,5] Cyclosporine A (CsA) inhibits TCR-mediated activation and IL-2 production, whereas rapamycin blocks intracellular signaling in response to T-cell growth factors like IL-2.[6,7] It can therefore be expected that CsA and rapamycin have different effects on the function of $CD4^+CD25^+$ T_{REG}s. Indeed, calcineurin inhibition by CsA may impair the development and function of T_{REG}s, whereas rapamycin was found to favor $CD4^+CD25^+$ T-cell–dependent immunoregulation in vitro and in vivo.[8-12]

Recently, we reported that allogeneic expansion of human naturally occurring $CD4^+CD25^+$ T_{REG}s leads to the emergence of a distinct, highly suppressive $CD27^+T_{REG}$ subset next to a $CD27^-T_{REG}$ subset.[13] These $CD27^+T_{REG}$ and $CD27^-T_{REG}$ subsets displayed a 50% inhibition of in vitro responses at ratios of 1:500 and 1:50, respectively, and were further distinguished by distinct growth characteristics and phenotype. The $CD27^+T_{REG}$ subset was shown to suppress not only naive and antigen-experienced memory T cells but also ongoing T-cell responses. In line with our findings, $CD4^+CD25^+CD27^+$ T_{REG}s were identified as a potent regulatory subset in cord blood and in a large-scale in vitro expansion system.[14,15] In addition, the combined expression of CD25 and CD27 allowed the differentiation of highly suppressive $FoxP3^+$ regulatory T cells from activated effector T cells in synovial fluid of patients with juvenile idiopathic arthritis.[16] It can be envisaged that the strong regulatory capacity of $CD27^+T_{REG}$ may be of value in promoting stable allograft tolerance.

Here, we describe that both CsA and rapamycin permit the activation of the naturally occurring regulatory $CD4^+CD25^+$ T_{REG}. However, rapamycin fosters the dominance of $CD27^+T_{REG}$ over $CD27^-T_{REG}$ after expansion of the $CD4^+CD25^+$ T_{REG} pool on allogeneic activation, whereas expansion in the presence of CsA tips the balance in favor of $CD27^-T_{REG}$. The high $CD27^+T_{REG}/CD27^-T_{REG}$ subset ratio, as preserved by rapamycin, benefits the suppressive capacity of the $CD4^+CD25^+$ T_{REG} pool as a whole. Moreover, although T_{REG}s cultured in the presence of either CsA or rapamycin are able to suppress naive T-cell responses, only T_{REG}s cultured in the presence of rapamycin can suppress ongoing T-cell responses. We thus propose that rapamycin promotes the induction of allograft tolerance by preserving a beneficial $CD27^+T_{REG}/CD27^-T_{REG}$ subset ratio.

From the Department of Bloodtransfusion and Transplantation Immunology and the Department of Nephrology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands.

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J.J.A.C. and H.J.P.M.K. contributed equally to this study.

Reprints: Irma Joosten, Department of Bloodtransfusion and Transplantation Immunology, Radboud University Nijmegen Medical Centre, PO Box 9101, 6500 HB Nijmegen, The Netherlands; e-mail: i.joosten@abti.umcn.nl.

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Materials and methods

The protocols of this study were performed in accordance with the Declaration of Helsinki, and were approved by the Institutional Review Board of the Radboud University Medical Center in Nijmegen, the Netherlands. Voluntary blood donors gave written informed consent.

Isolation of cells

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Lymphoprep; Nycomed Pharma, Oslo, Norway) of buffy-coats obtained from healthy donors. $CD4^+$ T cells were purified from PBMCs by negative selection using monoclonal antibodies (mAb's) directed against CD8 (RPA-T8), CD14 (M5E2), CD16 (3G8), CD19 (4G7), CD33 (P67.6), CD56 (B159), and CD235 (BD Biosciences, Erembodegem, Belgium) combined with sheep anti–mouse IgG–coated magnetic beads (Dynal Biotech, Oslo, Norway). This resulted in a $CD4^+$ T-cell purity of greater than 95% and the absence of $CD8^+$ T cells. From purified $CD4^+$ T cells, naturally occurring $CD4^+CD25^+$ T cells were isolated using the magnetic-activated cell sorting (MACS) $CD25^+$ magnetic microbead method (Miltenyi Biotec, Bergisch Gladbach, Germany), using half the amount of beads recommended by the manufacturer. $CD4^+CD25^+$ T cells were immediately used after isolation, and all other cell types were used either fresh or on thawing of liquid nitrogen–stored cell stocks. HLA typing was conducted according to ASHI (American Society for Histocompatibility and Immunogenetics) standards and largely as described previously.[17]

Primary mixed lymphocyte reaction

Primary mixed lymphocyte reaction (MLR) cultures were performed by culturing 5×10^4 isolated $CD4^+CD25^+$ T cells or control $CD4^+CD25^-$ T cells with 0.5×10^5 fully HLA-mismatched γ-irradiated (30 Gy) stimulator PBMCs in 200 μL culture medium (RPMI-1640 with glutamax supplemented with 0.02 mM pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin [all from Gibco, Paisley, United Kingdom], and 10% human pooled serum [HPS]) at 37°C, 95% humidity, and 5% CO_2 in 96-well round-bottom plates (Greiner, Frickenhausen, Germany). Recombinant human IL-2 (12.5 U/mL; Proleukine; Chiron, Amsterdam, The Netherlands) and IL-15 (10 ng/mL; BioSource, Nivelles, Belgium) were added to the medium at the start of culture. Proliferation was analyzed by ^{3}H-thymidine incorporation using a Gas Scintillation Counter (Matrix 96 Beta-counter; Canberra Packard, Meriden, CT). To this end 0.037 MBq (1 μCi) ^{3}H-thymidine (ICN Pharmaceuticals, Irvine, CA) was added to each well, cells were harvested after 8 hours of culture, and ^{3}H-thymidine incorporation was measured. The ^{3}H-incorporation is expressed as mean plus or minus SD counts per 5 minutes of at least triplicate measurements.

CFSE labeling

T cells ($0.5\text{-}2 \times 10^6$) were labeled with 0.2 to 1 μM carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; Molecular Probes, Eugene, OR) just before stimulation. Intracellular esterases cleave the acetate groups leading to the fluorescent carboxyfluorescein succinimidyl ester (CFSE). Cell division accompanied by CFSE dilution was analyzed by flow cytometry.

Immunosuppressive drugs

Sandimmune cyclosporine A (CsA) was obtained from Novartis Pharma B.V. (Arnhem, The Netherlands). Rapamycin was kindly provided for research purposes (Dr S. N. Sehgal, Wyeth-Ayerst, NJ).

MLR coculture assay to study T-cell suppressor function

The suppressor capacity of T cells was studied in a MLR coculture assay. $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells were primed with (target) alloantigen in the presence of recombinant human IL-2 and IL-15. Following expansion, the cells were harvested, washed, and allowed to rest for 3 days in medium containing 2% to 5% HPS and 5 ng/mL IL-15. The cells of interest were added to a newly set-up primary MLR, consisting of freshly thawed original responder PBMCs and γ-irradiated (30 Gy) stimulator PBMCs.

Flow cytometry

Cells were phenotypically analyzed by 4- or 5-color flow cytometry as described previously.[17] The following conjugated mAbs were used: CD3 (UCHT1) PE, CD4 (MT310) PE, CD8 (DK25) PE, CD27 (M-T271) PE, CD25 (M-A251) PE (Beckman Coulter, Miami, FL), FoxP3 (PCH101) FITC (Ebioscience, San Diego, CA), CD4 (T4) ECD, CD4 (T4) PC5, CD25 (B1.49.9) PC5, and CD62L (DREG54) ECD (Beckman Coulter). Isotype-matched antibodies were used to define marker settings. Intracellular analysis of *CTLA4* and *FOX*P3 was performed after fixation and permeabilization, using Fix and Perm reagent (Ebioscience).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA), Microsoft Excel 2000 (Microsoft, Redman, WA), and Statistical Product and Services Solutions (SPSS) package version 12.0 (SPSS Science Chicago, IL). For comparisons between groups, we used, when appropriate, the Wilcoxon signed rank test or Student *t* test, assuming unequal variances because of small sample size. To determine the pooled standard deviation (Figure 6) we used the following formula: $SD_P = (((n_1 - 1)(SD_1)^2) + ((n_2 - 1)(SD_2)^2) / (n_1 + n_2 - 2))^{1/2}$.

Results

CsA and rapamycin both permit the activation and suppressor function of naturally occurring $CD4^+CD25^+$ T_{REG}s

T_{REG}s require activation to perform their immunoregulatory function. To asses whether CsA or rapamycin interfere in the allogeneic activation of suppressor function, we performed MLR coculture assays in the presence or absence of these drugs. Previous dose-response experiments indicated that CsA concentrations between 40 ng/mL and 400 ng/mL, and rapamycin concentrations between 10 nM and 1000 nM, are suboptimal for inhibition of the alloresponse of effector T cells.[18] Repetition of these experiments yielded the same results (data not shown). Therefore, the use of these concentrations of drugs would allow us to asses an additional inhibition of effector T cells by added T_{REG}s. Using this setup, we observed that the addition of $CD4^+CD25^+$ T_{REG}s to both CsA- and rapamycin-treated cultures led to an enhanced inhibition of $CD4^+CD25^-$ effector T-cell proliferation (Figure 1). Neither drug abrogated the suppressive effect of the added $CD4^+CD25^+$ T_{REG}s. It can thus be concluded that biologically active concentrations of both CsA and rapamycin allow the activation and subsequent suppressor function of $CD4^+CD25^+$ T_{REG}s.

CsA and rapamycin inhibit the alloantigen-driven expansion of $CD4^+CD25^+$ T_{REG}s

It can be envisaged that on activation of naturally occurring $CD4^+CD25^+$ T_{REG}s, the induction of tolerance in vivo is further promoted by subsequent antigen-specific expansion of cells with regulatory capacity. Indeed, experimental transplantation models have demonstrated that alloantigen-specific $CD4^+CD25^+$ T_{REG}s contribute significantly to transplantation tolerance.[19,20] Therefore, we analyzed the effects of CsA and rapamycin on allogeneic expansion of freshly isolated, naturally occurring human $CD4^+CD25^+$ T_{REG}s. To this end, freshly isolated $CD4^+CD25^+$ T cells were labeled with CFSE and stimulated with alloantigen and additional IL-2 and IL-15. These cytokines are known to drive

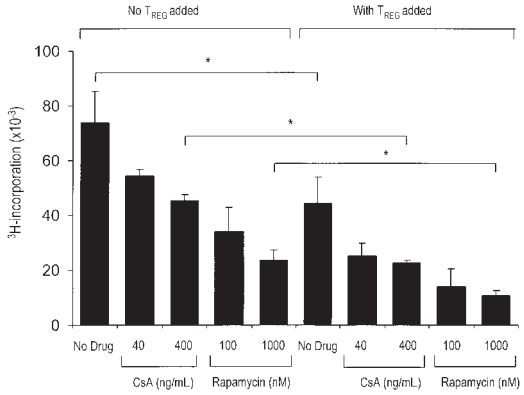

Figure 1. CsA and rapamycin both allow the activation and regulatory action of CD4$^+$CD25$^+$ T cells. Primary MLR was set up with 1×10^5 responder CD4$^+$CD25$^-$ T cells and 5×10^4 γ-irradiated (30 Gy) fully HLA-mismatched stimulator PBMCs. In coculture assays (right), CD4$^+$CD25$^+$ T cells were added in a suppressor-to-effector ratio of 1:4. CsA and rapamycin were added at the start of primary MLR at indicated concentrations (x axis). Proliferation at day 5 of culture as measured by ^{3}H-thymidine incorporation is shown on the y axis. One representative experiment is shown (n = 5). *$P < .05$ for difference between groups.

cell division in activated CD4$^+$CD25$^+$ T_{REG}s.[13] At day 6 of culture in the absence or presence of various doses of CsA or rapamycin, we analyzed the level of cell division by calculating the percentage of nondividing T cells (Figure 2). As anticipated, in the drug-free condition, CD4$^+$CD25$^+$ T_{REG}s proliferated strongly on fully allogeneic stimulation in the presence of IL-2 and IL-15, and only 36% of CD4$^+$CD25$^+$ T_{REG}s were left in the nondividing population. In contrast, both CsA and rapamycin induced a pronounced inhibition of cell division. In the case of the highest concentrations of the drugs tested, we observed a nondividing cell population of 62% for CsA and 64% for rapamycin. Taken together, both CsA and rapamycin are able to inhibit the expansion of CD4$^+$CD25$^+$ T_{REG}s on allogeneic activation in the presence of the T-cell growth factors IL-2 and IL-15.

In contrast to CsA, rapamycin fully preserves the regulatory capacity of expanded CD4$^+$CD25$^+$ T_{REG}s

Because antigen-driven expansion of CD4$^+$CD25$^+$ T_{REG}s has been demonstrated to increase the suppressive capacity of the resultant population,[13,21] a compromising effect of CsA or rapamycin on proliferation may weaken the regulatory function of the expanded population. To examine this, CD4$^+$CD25$^+$ T_{REG}s were stimulated with alloantigen and additional IL-2 and IL-15 in the absence or presence of CsA (400 ng/mL) or rapamycin (100 nM). Subsequently, the cells were washed, rested for 2 days, and tested for suppressive capacity in coculture MLR (as depicted in Figure 3A). Strikingly, CD4$^+$CD25$^+$ T_{REG}s cultured in the presence of rapamycin displayed potent dose-dependent suppressive capacity similar to control cells, whereas suppression by CD4$^+$CD25$^+$ T_{REG}s cultured in the presence of CsA was markedly reduced (Figure 3B).

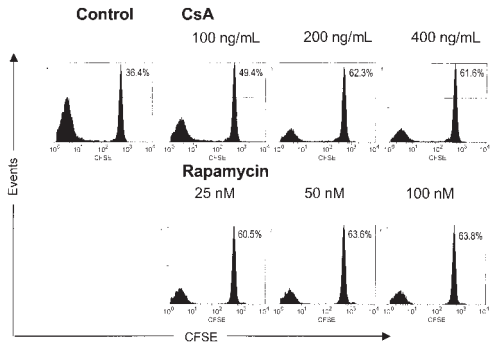

Figure 2. CsA and rapamycin inhibit the allogeneic expansion of CD4$^+$CD25$^+$ T cells. Freshly isolated CD4$^+$CD25$^+$ T cells (2.5×10^4) were labeled with CFSE and stimulated with γ-irradiated (30 Gy) HLA-mismatched stimulator PBMCs (1×10^5) in the presence of IL-2 and IL-15. CsA and rapamycin were added at the start of the cultures at indicated concentrations. Cell division represented by the dilution of CFSE was analyzed by flow cytometry at day 6 of culture. Histograms show CFSE intensity (x axis) and the number of events (y axis). One representative experiment is shown (n = 4).

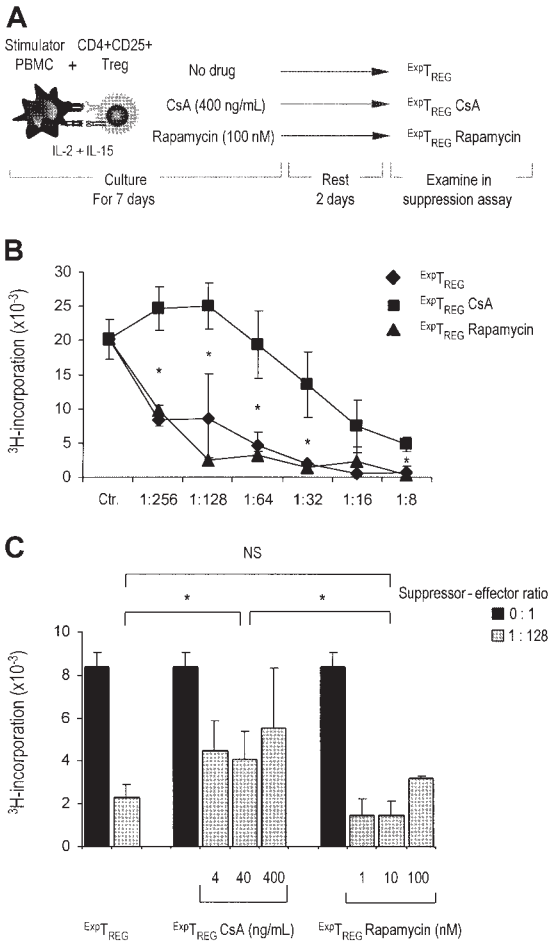

Figure 3. Rapamycin fosters regulatory function of expanded CD4$^+$CD25$^+$ T cells. (A) Schematic representation of CD4$^+$CD25$^+$ T-cell expansion culture. (B) Freshly isolated CD4$^+$CD25$^+$ T cells (2.5×10^4) were stimulated with 1×10^5 γ-irradiated (30 Gy) HLA-mismatched stimulator PBMCs and additional IL-2 and IL-15, in the absence or presence of CsA and rapamycin. CsA and rapamycin were added at the start of the cultures at indicated concentrations. Cells were harvested at day 7, washed 3 times, and rested. At day 10, the cells were examined for suppressor function in coculture assays. $^{Exp}T_{REG}$s derived from control (untreated), CsA-treated, and rapamycin-treated cultures were cocultured at the indicated suppressor-to-effector ratios (x axis) using 5×10^4 responder CD4$^+$CD25$^-$ effector T cells and 5×10^4 γ-irradiated (30 Gy) stimulator PBMCs. Proliferation at day 5 of culture as measured by ^{3}H-thymidine incorporation is shown on the y axis. One representative experiment is shown (n = 6). *$P < .05$ for difference between groups. (C) CD4$^+$CD25$^+$ T cells were cultured as described for panel B. CsA and rapamycin were added at the start of primary MLR at indicated concentrations (x axis). Cells were harvested at day 7, washed, rested, and cocultured at a suppressor-to-effector ratio of 1:128 with 5×10^4 responder CD4$^+$CD25$^-$ T cells and 5×10^4 γ-irradiated (30 Gy) stimulator PBMCs (▨). Control cultures were performed without the addition of suppressor cells (■). Proliferation at day 5 of culture, as measured by ^{3}H-thymidine incorporation, is shown on the y axis. One representative experiment of 3 independent experiments is shown. Because of small sample sizes, the conditions in which treated cells were used were grouped together, resulting in 3 groups: untreated cells, CsA-treated cells, and rapamycin-treated cells. *$P < .01$ for difference between groups. NS indicates not significant.

A 50% inhibition by T_{REG}s cultured in the presence of rapamycin was reached already at a suppressor-to-effector ratio of 1:256, whereas T_{REG}s cultured in the presence of CsA reached a 50% inhibition at a suppressor-to-effector ratio of 1:16. In other words, $CD4^+CD25^+$ T_{REG}s cultured in the presence of rapamycin were 16-fold more potent in suppressor capacity as compared with T_{REG} cultured in the presence of CsA. This difference was observed irrespective of the concentrations that were used, as shown in Figure 3C for a single $T_{REG}/T_{EFFECTOR}$ cell ratio. Thus, in contrast to CsA, rapamycin preserves suppressive capacity of T_{REG}s over a broad range of concentrations.

In contrast to CsA, rapamycin favors potent suppression by allowing a beneficial $CD27^+T_{REG}/CD27^-T_{REG}$ subset ratio

Previously, we demonstrated that after allogeneic expansion the $CD4^+CD25^+$ T_{REG} pool is composed of subsets of $CD27^-T_{REG}$s and extremely potent $CD27^+T_{REG}$s. On the basis of this observation and literature data[14-16] it is conceivable that the proportion of the highly potent $CD27^+T_{REG}$ subset relative to the $CD27^-T_{REG}$ subset will determine the overall suppressive capacity of the expanded T_{REG} pool. Therefore, we analyzed the proportions of $CD27^+T_{REG}$s and $CD27^-T_{REG}$s present in $CD4^+CD25^+$ T_{REG}s after allogeneic expansion in the presence of CsA or rapamycin. $CD4^+CD25^+$ T_{REG}s were cultured for 7 days in the presence of various concentrations of CsA or rapamycin. After 2 days of rest (ie, prior to putative addition to coculture MLR), cells were analyzed for the expression of CD25 and CD27. Interestingly, the T_{REG} pool cultured in the presence of rapamycin was, similar to control cells, dominated by the $CD27^+T_{REG}$ subset, whereas the T_{REG} pool cultured in the presence of CsA contained a relatively large proportion of $CD27^-T_{REG}$s (Figure 4A).

In six independent experiments, the $CD27^+T_{REG}/CD27^-T_{REG}$ subset ratios within the $CD25^+$ pool were determined after T_{REG} expansion in the absence or presence of immunosuppressive agents. It was found that T_{REG} expansion in the presence of CsA consistently resulted in a significant decrease of the $CD27^+T_{REG}/CD27^-T_{REG}$ subset ratio relative to T_{REG}s cultured in the presence of rapamycin or in the absence of either drug (Figure 4B, $P < .05$). Thus, expansion in the presence of rapamycin, and not CsA, preserves the highly potent $CD27^+T_{REG}$ subset.

Previously, $CD4^+CD25^+CD27^+$ T_{REG}s were characterized by a high *FOXP3* expression level.[16] Corresponding to the higher content of $CD27^+T_{REG}$s, $CD4^+CD25^+$ T_{REG}s cultured in the presence of rapamycin or in the absence of drugs displayed higher *FOXP3* expression than cells cultured in the presence of CsA (Figure 4C).

Subsequently, we analyzed the relationship between the $CD27^+T_{REG}/CD27^-T_{REG}$ subset ratio and the suppressive capacity for $CD4^+CD25^+$ T_{REG} populations cultured in the presence of CsA or rapamycin. In five of six experiments, the low $CD27^+T_{REG}/CD27^-T_{REG}$ subset ratio of T_{REG}s cultured in the presence of CsA corresponded to reduced suppressor potency. Conversely, when T_{REG}s were cultured in the presence of rapamycin or in the absence of drugs, a high $CD27^+T_{REG}/CD27^-T_{REG}$ subset ratio was related to a stronger suppressive activity (Figure 5).

In summary, the $CD27^+T_{REG}/CD27^-T_{REG}$ subset ratio crucially determines the suppressive capacity of the expanded T_{REG} pool as a whole. In contrast to CsA, rapamycin favors potent suppression by allowing a beneficial $CD27^+T_{REG}/CD27^-T_{REG}$ subset ratio.

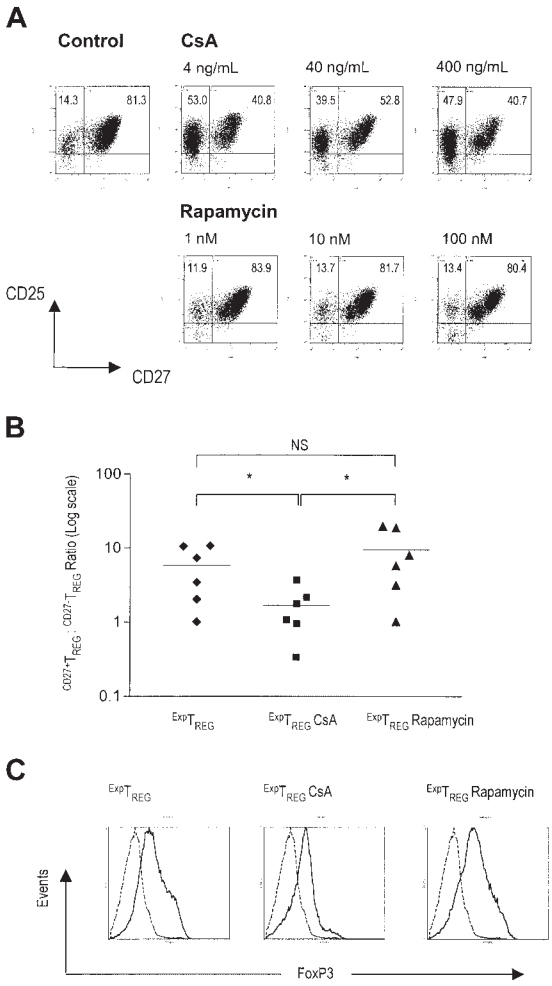

Figure 4. Rapamycin preserves a high $CD27^+T_{REG}/CD27^-T_{REG}$ ratio. (A) The expression of CD25 and CD27 on $CD4^+CD25^+$ T_{REG} expanded in the absence or presence of CsA or rapamycin was measured by flow cytometry. Freshly isolated $CD4^+CD25^+$ T cells (2.5×10^4) were stimulated with 1×10^5 γ-irradiated (30 Gy) allogeneic PBMCs and additional IL-2 and IL-15. CsA and rapamycin were added at the start of primary MLR at indicated concentrations. Cells were harvested at day 7, washed, rested, and analyzed for CD25 and CD27 expression at day 10 of culture. Dot plots show CD25 and CD27 expression of live-gated cells. One representative experiment of 3 independent experiments is shown. (B) $CD27^+T_{REG}/CD27^-T_{REG}$ subset ratios (y axis) within the $CD25^+$ fraction were calculated for the $^{Exp}T_{REG}$ pool, the $^{Exp}T_{REG}$ CsA pool (cultured with 400 ng/mL CsA), and the $^{Exp}T_{REG}$ rapamycin pool (cultured with 100 nM rapamycin). The results of 6 independent experiments are shown. $^*P < .05$ for difference between groups. (C) The intracellular expression profile of *FOXP3* in the $^{Exp}T_{REG}$ pool, the $^{Exp}T_{REG}$ CsA pool (cultured with 400 ng/mL CsA), and the $^{Exp}T_{REG}$ rapamycin pool (cultured with 100 nM rapamycin) was determined by flow cytometry. Histograms show intracellular *FOXP3* expression of live-gated cells. Dotted line indicates $CD4^+CD25^-$ cells; solid line, $CD4^+CD25^+$ cells.

Only $CD4^+CD25^+$ T_{REG}s cultured in the presence of rapamycin inhibit ongoing T-cell responses

In patients who receive a transplant and who have been primed with alloantigens, or in patients presenting with T-cell–mediated autoimmune diseases, it is crucial to restrain T-cell responses that are already in progress. As described previously, a crucial difference between $CD27^+T_{REG}$s and $CD27^-T_{REG}$s is the ability of $CD27^+T_{REG}$s to inhibit ongoing T-cell responses. Because rapamycin, but not CsA, preserves the $CD27^+T_{REG}$ pool, it can be hypothesized that T_{REG}s cultured in the presence of either drug differ with respect to their ability to inhibit ongoing T-cell responses. Therefore, we analyzed the potential of T_{REG}s cultured in the presence of immunosuppressive drugs to inhibit effector

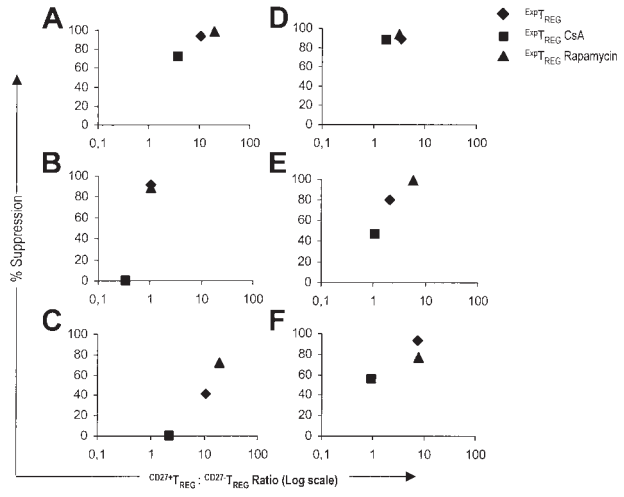

Figure 5. A high $CD27^+T_{REG}/CD27^-T_{REG}$ ratio corresponds to potent suppression. The relative suppression (y axis) at a suppressor-to-effector ratio of 1:128 was plotted against the corresponding $CD27^+T_{REG}/CD27^-T_{REG}$ subset ratio (x axis) for the $^{Exp}T_{REG}$ pool (◆), the $^{Exp}T_{REG}$ CsA pool (cultured with 400 ng/mL CsA) (■), and the $^{Exp}T_{REG}$ rapamycin pool (cultured with 100 nM rapamycin) (▲). The results of 6 independent experiments are shown.

T-cell proliferation at an advanced state of a MLR. Notably, T_{REG}s cultured in the presence of CsA lost the ability to prevent ongoing T-cell responses of 2 days after the start of MLR, whereas T_{REG}s cultured in the presence of rapamycin were still able to suppress this response (Figure 6).

Discussion

In several experimental transplantation models, it has been observed that CsA can antagonize the induction of tolerance, whereas rapamycin does not.[22-25] One mechanism that may account for these observations is that in contrast to CsA, rapamycin easily permits apoptosis of alloreactive T cells.[8] Another possibility is that both drugs have different effects on T_{REG}s because these cells have been demonstrated to play an important role in many models of tolerance induction.[9] Indeed, it was reported that CsA, and not rapamycin, blocks the generation of T_{REG}s after pretransplantation donor-specific blood transfusion in rats.[12] Furthermore, a relative increase of $CD4^+CD25^+$ T cells in peripheral lymphoid organs was observed in rats treated with rapamycin. Recently, Battaglia et al[11] described that exposure to rapamycin results in a selective expansion of murine $CD4^+CD25^+$ T_{REG}s in vitro.

We here present a novel explanation for the favorable immunomodulatory effects of rapamycin. First of all, we confirm that both rapamycin and CsA allow the activation and suppressor function of human T_{REG}s. As a new finding, we demonstrate that after allogeneic stimulation of human naturally occurring $CD4^+CD25^+$ T_{REG}s, rapamycin treatment fosters a high $CD27^+T_{REG}/CD27^-T_{REG}$ subset ratio. Recently, we described the characteristics of these 2 T_{REG} subsets induced on allogeneic expansion of human $CD4^+CD25^+$ T_{REG}s.[13] A major difference between these subsets is their suppressive capacity, the $CD27^+T_{REG}$ subset being highly suppressive, whereas the $CD27^-T_{REG}$ subset has modest suppressive potency.[13] The strong regulatory capacity of the $CD27^+T_{REG}$ subset has also been shown in other studies.[14-16] After expansion of $CD4^+CD25^+$ T_{REG}s, we typically find that the majority of cells are $CD27^+$. In this study we show that rapamycin and CsA act differently with regard to the preservation of the high $CD27^+T_{REG}/CD27^-T_{REG}$ ratio. In contrast to CsA, rapamycin preserved the dominance of $CD27^+T_{REG}$s over $CD27^-T_{REG}$s on allogeneic stimulation, resulting in strong suppressive capacity of the expanded $CD4^+CD25^+$ T_{REG} pool as a whole. It can thus be envisaged that a predominance of $CD27^+T_{REG}$s, which is supported by treatment with rapamycin, can add to the development of stable tolerance and prevention of rejection in solid-organ transplantation. Also in stem cell transplantation, patients may benefit from the use of rapamycin as immunosuppressant, for it becomes increasingly clear that the presence of potent $CD4^+CD25^+$ T_{REG}s favors immune reconstitution and may control graft-versus-host disease while allowing graft-versus-tumor activity.[26,27]

Next to the beneficial effect of $CD27^+T_{REG}$s, the relevance of the $CD27^-T_{REG}$s as such should not be discarded. It can be expected that after transplantation $CD4^+CD25^+$ T_{REG}s will expand in an antigen-specific manner in the draining lymph nodes or in the graft.[28,29] Of note, $CD27^+T_{REG}$s and $CD27^-T_{REG}$s differ with respect to the pattern of migratory receptors and adhesion molecules. $CD27^+T_{REG}$s were found to express CD62L, whereas $CD27^-T_{REG}$s were devoid of CD62L.[13] It can thus be argued that $CD27^+T_{REG}$s are committed to local suppressor function in the draining lymph node, whereas $CD27^-T_{REG}$s are destined to enter the periphery. Moreover, $CD27^-T_{REG}$s are characterized by rapid expansion, which may be of importance in outnumbering aggressive T cells in the periphery.[13] So, $CD27^-T_{REG}$s may have a specific role, and in vivo it may well be that the induction of transplantation tolerance benefits from the use of CsA in specific cases. In fact, CsA has been implicated in the induction of transplantation tolerance by sparing of $CD4^+$ suppressor cells and when used in combination with costimulation blocking agents.[12,30-34] Further research on the in vivo role of $CD27^+T_{REG}$s and $CD27^-T_{REG}$s may elucidate how the effect of rapamycin and CsA on the skewing of T_{REG} subsets may differentially affect the induction of allograft tolerance.

The frequency of $CD27^+T_{REG}$s is clearly affected by CsA. Crosslinking of CD27 has been shown to induce proliferation following the mobilization of intracellular free Ca^{2+}.[35,36] Because CsA specifically targets Ca^{2+}-dependent activation pathways, CsA may thus selectively inhibit $CD27^+T_{REG}$ proliferation. However, we have preliminary data that do not support an inhibition of

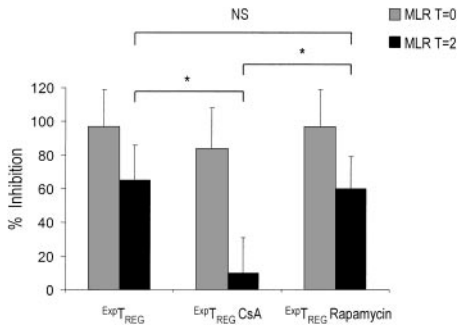

Figure 6. $CD4^+CD25^+$ T cells cultured in the presence of rapamycin, but not CsA, are able to inhibit ongoing T-cell responses. Freshly isolated $CD4^+CD25^+$ T cells (2.5×10^4) were stimulated with 1×10^5 γ-irradiated (30 Gy) HLA-mismatched stimulator PBMCs and additional IL-2 and IL-15 in the absence or presence of CsA (400 ng/mL) and rapamycin (100 nM). Cells were harvested at day 7, washed 3 times, and rested. At day 10, the cells were examined for suppressor function in suppression coculture assays. $^{Exp}T_{REG}$s derived from control (untreated), CsA-treated, and rapamycin-treated cultures (x axis) were cocultured from day 0 (T = 0) (▨) or day 2 (T = 2) (■) of MLR at a suppressor-to-effector ratio of 1:16 using 5×10^4 responder $CD4^+CD25^-$ effector T cells and 5×10^4 γ-irradiated (30 Gy) stimulator PBMCs. Proliferation at day 5 of culture was measured by ^{3}H-thymidine incorporation and is depicted as a percentage of inhibition on the y axis. A representative experiment is shown. *$P < .05$ for difference between groups.

proliferation (data not shown), rather it appears that CD27 expression levels are affected on stimulation in the presence of CsA. This is in agreement with the previous finding that elevation of intracellular Ca^{2+} levels is important in the up-regulation of CD27 expression.[36]

It has been proposed that signaling by the growth factor IL-2 is crucial for the functional activity of T_{REG}s.[4,5] CsA inhibits TCR-induced activation and IL-2 production, whereas rapamycin blocks signaling in response to T-cell growth factors.[6,7] In theory, both drugs could therefore interfere with the function of T_{REG} function. Interestingly, we observed that in the presence of CsA or rapamycin freshly isolated $CD4^+CD25^+$ T_{REG}s were able to suppress $CD4^+CD25^-$ T cells, indicating that CsA and rapamycin did not significantly interfere in the activation or suppressor function of these cells. In line with these findings, basiliximab, a chimeric monoclonal antibody directed against the IL-2 receptor, also did not interfere in the suppressive capacity of human $CD4^+CD25^+$ T cells.[37] This may indicate that inhibition of the IL-2 pathways is not as detrimental to the function of human $CD4^+CD25^+$ T_{REG}s as could be expected from in vivo findings on $CD4^+CD25^+$ T_{REG}s in a mouse model.[38]

In summary, our data show that CsA and rapamycin do not interfere with the activation or suppressor function of freshly isolated human $CD4^+CD25^+$ T_{REG}s. However, CsA and rapamycin differentially affect T_{REG}-subset heterogeneity on expansion after allogeneic activation. Expansion of $CD4^+CD25^+$ T_{REG}s in the presence of rapamycin favors $CD27^+T_{REG}$-subset dominance, which is beneficial for the suppressive capacity of the $CD4^+CD25^+$ T-cell pool as a whole and allows the suppression of ongoing T-cell responses. During expansion of T_{REG}s in the presence of CsA, the dominance of the $CD27^+T_{REG}$ subset is lost, and this is accompanied by a decrease of suppressive activity of the resulting population. These findings provide a novel contribution to explain the favorable effects of rapamycin in strategies of tolerance induction.

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