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

Running title
Rapamycin preserves CD4+CD25+CD27+ T-cells

Authors
Jeroen J.A. Coenen1,2, Hans J.P.M. Koenen1, Esther van Rijssen1, Luuk B. Hilbrands2, and Irma Joosten1

Institution where work was performed
1Dept. of Bloodtransfusion and Transplantation Immunology and 2Dept. of Nephrology, Radboud University Nijmegen Medical Centre, PO Box 9101, 6500 HB, Nijmegen, The Netherlands.

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Footnote:
JC and HK contributed equally to this paper

Corresponding author
Dr. I. Joosten
Department of Bloodtransfusion and Transplantation Immunology
Radboud University Nijmegen Medical Centre
P.O. Box 9101, 6500 HB Nijmegen
The Netherlands
Phone: ++31 24 3615335
Fax: ++31 24 3619415
E-mail: i.joosten@abti.umcn.nl
Abstract

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\textsubscript{REG}) population, which plays an important role in allograft tolerance. Recently, we reported that alloantigen driven expansion of human CD4+CD25+ T\textsubscript{REG} gives rise to a distinct highly suppressive CD27+T\textsubscript{REG} subset next to a moderately suppressive CD27-T\textsubscript{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\textsubscript{REG} subset over the CD27-T\textsubscript{REG} subset after alloantigen driven expansion of CD4+CD25+ T\textsubscript{REG} in vitro. Accordingly, CD4+CD25+ T\textsubscript{REG} cultured in the presence of rapamycin displayed much stronger suppressive capacity than CD4+CD25+ T\textsubscript{REG} cultured in the presence of CsA. In addition, CD4+CD25+ T\textsubscript{REG} 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\textsubscript{REG} subset dominance, may favor the use of rapamycin in tolerance-inducing strategies.
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})\(^1,2\). 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} 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}\(^4,5\). Cyclosporine (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}. Indeed, calcineurin inhibition by CsA may impair the development and function of T_{REG}, whereas rapamycin was found to favor CD4⁺CD25⁺ T cell dependent immunoregulation \textit{in vitro} and \textit{in vivo}\(^8-12\).

Recently, we reported that allogeneic expansion of human naturally occurring CD4⁺CD25⁺ T_{REG} 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 \textit{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 naïve and antigen experienced memory T cells, but also ongoing T cell responses. In line with our findings, CD4⁺CD25⁺CD27⁺ T_{REG} were identified as a potent regulatory subset in cord blood and in a large scale \textit{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 juvenile idiopathic arthritis patients\(^16\). 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 upon allogeneic activation, whereas expansion in the presence of CsA tips the balance in favor of CD27^-T_{REG}. The high CD27^+T_{REG} to CD27^-T_{REG} subset ratio, as preserved by rapamycin, benefits the suppressive capacity of the CD4^+CD25^+ T_{REG} pool as a whole. Moreover, while T_{REG} cultured in the presence of either CsA or rapamycin are able to suppress naïve T cell responses, only T_{REG} 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} to CD27^-T_{REG} subset ratio.
Materials and methods

Isolation of cells

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (Lymphoprep, Nycomed Pharma AS, Oslo, Norway) of buffy-coats obtained from normal healthy donors. CD4+ T cells were purified from PBMC by negative selection using monoclonal antibodies (mAb) 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 >95% and the absence of CD8+ T cells. From purified CD4+ T cells, naturally occurring CD4+CD25+ T cells were isolated using the MACS CD25+ magnetic microbead method (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), using half the amount of beads recommended by the manufacturer. CD4+CD25+ T cells were immediately used after isolation, while all other cell types were used either fresh or upon 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 previously17.

Primary Mixed Lymphocyte Reaction

Primary Mixed Lymphocyte Reaction (MLR) cultures were performed by culturing 5x10^4 isolated CD4+CD25+ T cells or control CD4+CD25- T cells with 0.5x10^5 fully HLA mismatched γ-irradiated (30 Gy) stimulator PBMC in 200 μl culture medium (RPMI-1640 with glutamax supplemented with (0.02mM) pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin (all Gibco, Paisley, UK), and 10% human pooled serum (HPS)) at 37°C, 95% humidity and 5% CO2 in 96 wells round bottom plates (Greiner, Frickenhausen, Germany). Recombinant human IL-2 (12.5 U/ml, Proleukine, 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 ³H-thymidine incorporation using a Gas Scintillation Counter (Canberra Packard,
Matrix 96 Beta-counter, Meriden, U.S.A.). To this end 0.037MBq (1μCi) $^3$H-thymidine (ICN Pharmaceuticals, Irvine, CA, USA) 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 ±SD counts per 5 minutes of at least triplicate measurements.

**CFSE labeling**

T cells (0.5-2 x10$^6$) were labeled with 0.2-1 μM Carboxyfluorescein Diacetate Succinimidyl Ester (CFDA-SE, Molecular Probes, Eugene, OR, USA) 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 flowcytometry.

**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, USA).

**MLR co-culture assay to study T cell suppressor function**

The suppressor capacity of T cells was studied in a MLR co-culture 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, allowed to rest for 3 days in medium containing 2-5% 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 PBMC and $\gamma$-irradiated (30 Gy) stimulator PBMC.
Flowcytometry

Cells were phenotypically analyzed by four or five color flowcytometry as described previously\textsuperscript{17}. The following conjugated mAb were used: CD3 (UCHT1) PE, CD4 (MT310) PE, CD8 (DK25) PE, CD27 (M-T271) PE, CD25 (M-A251) PE, (Beckman Coulter Corporation, Miami, Fl, USA), 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 CTLA-4 and FoxP3 was performed after fixation and permeabilization, using Fix and Perm reagent (Ebioscience, San Diego, CA).

Statistical analysis

Statistical Analysis was performed using Graphpad Prism 3.0, Microsoft Excel 2000 and Statistical Product and Services Solutions (SPSS) package version 12.0. For comparisons between groups, we used where appropriate, the Wilcoxon signed rank test or Student’s t test, assuming unequal variances because of small sample size. To determine the pooled standard deviation (Fig. 6) we used the following formula;

\[ SD_p = \sqrt{\frac{(n_1-1)(SD_1)^2 + (n_2-1)(SD_2)^2}{(n_1+n_2-2)}}. \]
Results

CsA and rapamycin both permit the activation and suppressor function of naturally occurring CD4+CD25+ TREG

TREG require activation in order to perform their immunoregulatory function. To assess whether CsA or rapamycin interfere in the allogeneic activation of suppressor function, we performed MLR co-culture 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 alloreponse of effector T cells. Repetition of these experiments yielded the same results (data not shown). Therefore, the use of these concentrations of drugs would allow us to assess an additional inhibition of effector T cells by added TREG. Using this set-up, we observed that the addition of CD4+CD25+ TREG to both CsA and rapamycin treated cultures led to an enhanced inhibition of CD4+CD25- effector T-cell proliferation (Fig. 1). Neither drug abrogated the suppressive effect of the added CD4+CD25+ TREG. It can thus be concluded that biologically active concentrations of both CsA and rapamycin allow the activation and subsequent suppressor function of CD4+CD25+ TREG.

CsA and rapamycin inhibit the alloantigen driven expansion of CD4+CD25+ TREG

It can be envisaged that upon activation of naturally occurring CD4+CD25+ TREG 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+ TREG contribute significantly to transplantation tolerance. Therefore, we analyzed the effects of CsA and rapamycin on allogeneic expansion of freshly isolated human naturally occurring CD4+CD25+ TREG. 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 cell division in activated CD4+CD25+ regulatory T-cells. 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 non-dividing T cells (Fig. 2). As anticipated, in the drug-free condition CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> proliferated strongly upon fully allogeneic stimulation in the presence of IL-2 and IL-15, and only 36% of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> was left in the non-dividing population. In contrast, both CsA and rapamycin induced a pronounced inhibition of cell division. In case of the highest concentrations of the drugs tested we observed a non-dividing cell population of 62% for CsA and 64% for rapamycin. Taken together, both CsA and rapamycin are able to inhibit the expansion of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> upon 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<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub>

Since antigen driven expansion of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> has been demonstrated to increase the suppressive capacity of the resultant population<sup>13,21</sup>, a compromising effect of CsA or rapamycin on proliferation may weaken the regulatory function of the expanded population. To examine this, CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> 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 two days, and tested for suppressive capacity in co-culture MLR (as depicted in Fig. 3A). Strikingly, CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cultured in the presence of rapamycin displayed potent dose-dependent suppressive capacity similar to control cells, whereas suppression by CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cultured in the presence of CsA was markedly reduced (Fig. 3B). A 50% inhibition by T<sub>REG</sub> cultured in the presence of rapamycin was reached already at a suppressor:effector ratio of 1:256, whereas T<sub>REG</sub> cultured in the presence of CsA reached a 50% inhibition at a suppressor:effector ratio of 1:16. In other words, CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cultured in the presence of rapamycin were 16-fold more potent in suppressor capacity as compared to Treg 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 $T_{\text{REG}}$ to $T_{\text{EFFECTOR}}$ cell ratio. Thus, in contrast to CsA, rapamycin preserves suppressive
capacity of $T_{\text{REG}}$ over a broad range of concentrations.
In contrast to CsA, rapamycin favors potent suppression by allowing a beneficial $\text{CD}^{27+}\text{T}_{\text{REG}}$ to $\text{CD}^{27-}\text{T}_{\text{REG}}$ subset ratio.

Previously, we demonstrated that after allogeneic expansion the CD4$^+$CD25$^+$ T$_{\text{REG}}$ pool is composed of subsets of $\text{CD}^{27-}\text{T}_{\text{REG}}$ and extremely potent $\text{CD}^{27+}\text{T}_{\text{REG}}$. Based upon this observation and literature data$^{14-16}$ it is conceivable that the proportion of the highly potent $\text{CD}^{27+}\text{T}_{\text{REG}}$ subset relative to the $\text{CD}^{27-}\text{T}_{\text{REG}}$ subset will determine the overall suppressive capacity of the expanded T$_{\text{REG}}$ pool. Therefore, we analyzed the proportions of $\text{CD}^{27+}\text{T}_{\text{REG}}$ and $\text{CD}^{27-}\text{T}_{\text{REG}}$ present in CD4$^+$CD25$^+$ T$_{\text{REG}}$ after allogeneic expansion in the presence of CsA or rapamycin. CD4$^+$CD25$^+$ T$_{\text{REG}}$ were cultured for 7 days in the presence of various concentrations of CsA or rapamycin. After two days of rest, i.e. prior to putative addition to co-culture MLR, cells were analyzed for the expression of CD25 and CD27. Interestingly, the T$_{\text{REG}}$ pool cultured in the presence of rapamycin was, similar to control cells, dominated by the $\text{CD}^{27+}\text{T}_{\text{REG}}$ subset, whereas the T$_{\text{REG}}$ pool cultured in the presence of CsA contained a relatively large proportion of $\text{CD}^{27-}\text{T}_{\text{REG}}$ (Fig. 4A).

In six independent experiments, the $\text{CD}^{27+}\text{T}_{\text{REG}}$ to $\text{CD}^{27-}\text{T}_{\text{REG}}$ subset ratios within the CD25$^+$ pool were determined after T$_{\text{REG}}$ expansion in the absence or presence of immunosuppressive agents. It was found that T$_{\text{REG}}$ expansion in the presence of CsA consistently resulted in a significant decrease of the $\text{CD}^{27+}\text{T}_{\text{REG}}$ to $\text{CD}^{27-}\text{T}_{\text{REG}}$ subset ratio relative to T$_{\text{REG}}$ cultured in the presence of rapamycin or in the absence of either drug (Fig. 4B, p<0.05). Thus, expansion in the presence of rapamycin, and not CsA, preserves the highly potent $\text{CD}^{27+}\text{T}_{\text{REG}}$ subset.

Previously, the CD4$^+$CD25$^+$CD27$^+$ T$_{\text{REG}}$ was characterized by a high FoxP3 expression level$^{16}$. Corresponding to the higher content of $\text{CD}^{27+}\text{T}_{\text{REG}}$, CD4$^+$CD25$^+$ T$_{\text{REG}}$ cultured in the presence of rapamycin or in the absence of drugs displayed higher FoxP3 expression than cells cultured in the presence of CsA (Fig. 4C).

Subsequently, we analyzed the relationship between the $\text{CD}^{27+}\text{T}_{\text{REG}}$ to $\text{CD}^{27-}\text{T}_{\text{REG}}$ subset ratio and the suppressive capacity for CD4$^+$CD25$^+$ T$_{\text{REG}}$ populations cultured in the presence of CsA or rapamycin. In five out of six experiments the low $\text{CD}^{27+}\text{T}_{\text{REG}}$ to $\text{CD}^{27-}\text{T}_{\text{REG}}$ subset ratio
of T\textsubscript{REG} cultured in the presence of CsA corresponded to reduced suppressor potency. Conversely, when T\textsubscript{REG} were cultured in the presence of rapamycin or in the absence of drugs, a high CD\textsuperscript{27+}\textsubscript{T\textsubscript{REG}} to CD\textsuperscript{27-}\textsubscript{T\textsubscript{REG}} subset ratio was related to a stronger suppressive activity (Fig. 5).

In summary, the CD\textsuperscript{27+}\textsubscript{T\textsubscript{REG}} to CD\textsuperscript{27-}\textsubscript{T\textsubscript{REG}} subset ratio crucially determines the suppressive capacity of the expanded T\textsubscript{REG} pool as a whole. In contrast to CsA, rapamycin favors potent suppression by allowing a beneficial CD\textsuperscript{27+}\textsubscript{T\textsubscript{REG}} to CD\textsuperscript{27-}\textsubscript{T\textsubscript{REG}} subset ratio.

Only CD\textsuperscript{4+}\textsuperscript{CD25+} T\textsubscript{REG} cultured in the presence of rapamycin inhibit ongoing T-cell responses.

In transplant patients that 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 CD\textsuperscript{27+}\textsubscript{T\textsubscript{REG}} and CD\textsuperscript{27-}\textsubscript{T\textsubscript{REG}} is the ability of CD\textsuperscript{27+}\textsubscript{T\textsubscript{REG}} to inhibit ongoing T cell responses. Since rapamycin, but not CsA, preserves the CD\textsuperscript{27+}\textsubscript{T\textsubscript{REG}} pool it can be hypothesized that T\textsubscript{REG} 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\textsubscript{REG} cultured in the presence of immunosuppressive drugs to inhibit effector T cell proliferation at an advanced state of a MLR. Notably, T\textsubscript{REG} 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\textsubscript{REG} cultured in the presence of rapamycin were still able to suppress this response (Fig. 6).
Discussion

In several experimental transplantation models it has been observed that CsA can antagonize the induction of tolerance, while rapamycin does not\textsuperscript{22-25}. One mechanism that may account for these observations is that in contrast to CsA, rapamycin easily permits apoptosis of alloreactive T cells\textsuperscript{8}. Another possibility is that both drugs have different effects on T\textsubscript{REG} since these cells have been demonstrated to play an important role in many models of tolerance induction\textsuperscript{9}. Indeed, it was reported that CsA, and not rapamycin, blocks the generation of T\textsubscript{REG} after pretransplant donor-specific blood transfusion in rats\textsuperscript{12}. Furthermore, a relative increase of CD4\textsuperscript{+}CD25\textsuperscript{+} T cells in peripheral lymphoid organs was observed in rats treated with rapamycin. Recently, Battaglia et al.\textsuperscript{11} described that exposure to rapamycin results in a selective expansion of murine CD4\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{REG} \textit{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\textsubscript{REG}. As a new finding, we demonstrate that after allogeneic stimulation of human naturally occurring CD4\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{REG}, rapamycin treatment fosters a high CD27\textsuperscript{+}T\textsubscript{REG} to CD27\textsuperscript{-}T\textsubscript{REG} subset ratio. Recently, we described the characteristics of these two T\textsubscript{REG} subsets induced upon allogeneic expansion of human CD4\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{REG}\textsuperscript{13}. A major difference between these subsets is their suppressive capacity, the CD27\textsuperscript{+}T\textsubscript{REG} subset being highly suppressive, while the CD27\textsuperscript{-}T\textsubscript{REG} subset has modest suppressive potency\textsuperscript{13}. The strong regulatory capacity of the CD27\textsuperscript{+}T\textsubscript{REG} subset has also been shown in other studies\textsuperscript{14-16}. After expansion of CD4\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{REG}, we typically find that the majority of cells is CD27\textsuperscript{+}. In this study we show that rapamycin and CsA act differently with regard to the preservation of the high CD27\textsuperscript{+}T\textsubscript{REG} to CD27\textsuperscript{-}T\textsubscript{REG} ratio. In contrast to CsA, rapamycin preserved the dominance of CD27\textsuperscript{+}T\textsubscript{REG} over CD27\textsuperscript{-}T\textsubscript{REG} upon allogeneic stimulation, resulting in strong suppressive capacity of the expanded CD4\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{REG} pool as a whole. It can thus be envisaged that a predominance of CD27\textsuperscript{+}T\textsubscript{REG}, 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⁺ regulatory T-cells favors immune reconstitution and may control graft-versus-host disease while allowing graft-versus-tumor activity²⁶,²⁷.

Next to the beneficial effect of CD27⁺Treg, the relevance of the CD27⁻TREG as such should not be discarded. It can be expected that after transplantation CD4⁺CD25⁺ TREG will expand in an antigen-specific manner in the draining lymph nodes or in the graft²⁸,²⁹. Of note, CD27⁺TREG and CD27⁻TREG differ with respect to the pattern of migratory receptors and adhesion molecules. CD27⁺TREG were found to express CD62L, whereas CD27⁻TREG were devoid of CD62L.³⁰ It can thus be argued that CD27⁺TREG are committed to local suppressor function in the draining lymph node, whereas CD27⁻TREG are destined to enter the periphery. Moreover, CD27⁻TREG are characterized by rapid expansion, which may be of importance in outnumbering aggressive T-cells in the periphery.³¹ So, CD27⁻TREG 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 agents¹²,³⁰-³⁴. Further research on the in vivo role of CD27⁺TREG and CD27⁻TREG may elucidate how the effect of rapamycin and CsA on the skewing of TREG subsets may differentially affect the induction of allograft tolerance.

The frequency of the CD27⁺TREG is clearly affected by CsA. Cross-linking of CD27 has been shown to induce proliferation following the mobilization of intracellular free Ca²⁺³⁵,³⁶. Since CsA specifically targets Ca²⁺ dependent activation pathways, CsA may thus selectively inhibit CD27⁺TREG proliferation. However, we have preliminary data that do not support an inhibition of proliferation (data not shown), rather it appears that CD27 expression levels are affected upon stimulation in the presence of CsA. This is in agreement with the previous finding that elevation of intracellular Ca²⁺ 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 TREG.⁴,⁵ CsA inhibits TCR-induced activation and IL-2 production,
whereas rapamycin blocks signaling in response to T cell growth factors\textsuperscript{6,7}. In theory, both drugs could therefore interfere with the function of T\textsubscript{REG} function. Interestingly, we observed that in the presence of CsA or rapamycin freshly isolated CD4\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{REG} were able to suppress CD4\textsuperscript{+}CD25\textsuperscript{−} 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\textsuperscript{+}CD25\textsuperscript{+} T cells\textsuperscript{37}. This may indicate that inhibition of the IL-2 pathways is not as detrimental to the function of human CD4\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{REG} as could be expected from in vivo findings on CD4\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{REG} in a mouse model\textsuperscript{38}.

In summary, our data show that CsA and rapamycin do not interfere with the activation or suppressor function of freshly isolated human CD4\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{REG}. However, CsA and rapamycin differentially affect T\textsubscript{REG} subset heterogeneity upon expansion after allogeneic activation. Expansion of CD4\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{REG} in the presence of rapamycin favors CD27\textsuperscript{+}T\textsubscript{REG} subset dominance, which is beneficial for the suppressive capacity of the CD4\textsuperscript{+}CD25\textsuperscript{+} T cell pool as a whole and allows the suppression of ongoing T cell responses. During expansion of T\textsubscript{REG} in the presence of CsA, the dominance of the CD27\textsuperscript{+}T\textsubscript{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.
References


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Figure legends

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 PBMC. In co-culture assays (right-hand side of the figure), CD4^+CD25^+ T cells were added in a suppressor: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 ^3H-thymidine incorporation is shown on the Y-axis. One representative experiment is shown (n=5). *P<0.05 for difference between groups.

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 PBMC (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 flowcytometry 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. 2.5*10^4 freshly isolated CD4+CD25+ T-cells were stimulated with 1*10^5 γ-irradiated (30 Gy) HLA mismatched stimulator PBMC 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 co-culture assays. ExpTReg derived from control (untreated), CsA-treated, and rapamycin-treated cultures were co-cultured at the indicated suppressor:effector ratio’s (X-axis) using 5*10^4 responder CD4+CD25- effector T cells and 5*10^4 γ-irradiated (30 Gy) stimulator PBMC. Proliferation at day 5 of culture as measured by 3H-thymidine incorporation is shown on the Y-axis. One representative experiment is shown (n=6). *P<0.05 for difference between groups.

C. CD4+CD25+ T cells were cultured as described above. 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 co-cultured at a suppressor:effector ratio of 1:128 with 5*10^4 responder CD4+CD25- T cells and 5*10^4 γ-irradiated (30 Gy) stimulator PBMC (grey bars). Control cultures were performed without the addition of suppressor cells (black bars). Proliferation at day 5 of culture as measured by 3H-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 three groups; untreated cells, CsA treated cells and rapamycin treated cells. *P<0.01 for difference between groups.
Figure 4. Rapamycin preserves a high $^{\text{CD27}}_{\text{REG}}:^{\text{CD27}}_{\text{REG}}$ ratio.

A. The expression of CD25 and CD27 on CD4$^{+}$CD25$^{+}$ T$_{\text{REG}}$ expanded in the absence or presence of CsA or rapamycin was measured by flowcytometry. 2.5$\times$10$^4$ freshly isolated CD4$^{+}$CD25$^{+}$ T cells were stimulated with 1$\times$10$^5$ $\gamma$-irradiated (30 Gy) allogeneic PBMC 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 out of 3 independent experiments is shown.

B. $^{\text{CD27}}_{\text{REG}}$ to $^{\text{CD27}}_{\text{REG}}$ subset ratios (Y-axis) within the CD25$^{+}$ fraction were calculated for the $^{\text{Exp}}_{\text{T}_{\text{REG}}}$ pool, the $^{\text{Exp}}_{\text{T}_{\text{REG}}}$ CsA pool (cultured with 400 ng/mL CsA), and the $^{\text{Exp}}_{\text{T}_{\text{REG}}}$ rapamycin pool (cultured with 100 nM rapamycin). The results of six independent experiments are shown. *P<0.05 for difference between groups.

C. The intracellular expression profile of FoxP3 in the $^{\text{Exp}}_{\text{T}_{\text{REG}}}$ pool, the $^{\text{Exp}}_{\text{T}_{\text{REG}}}$ CsA pool (cultured with 400 ng/mL CsA) and the $^{\text{Exp}}_{\text{T}_{\text{REG}}}$ rapamycin pool (cultured with 100 nM rapamycin) was determined by flowcytometry. Histograms show intracellular FoxP3 expression of live-gated cells. *(Dotted line; CD4$^{+}$CD25$^{-}$ cells. Solid line; CD4$^{+}$CD25$^{+}$ cells).
**Figure 5. A high $^{CD27^+T_{REG}}:^{CD27^-T_{REG}}$ ratio corresponds to potent suppression**

The relative suppression (Y-axis) at a suppressor:effector ratio of 1:128 was plotted against the corresponding $^{CD27^+T_{REG}}$ to $^{CD27^-T_{REG}}$ subset ratio (X-axis) for the $^{ExpT_{REG}}$ pool, the $^{ExpT_{REG}}$ CsA pool (cultured with 400 ng/mL CsA) and $^{ExpT_{REG}}$ rapamycin pool (cultured with 100 nM rapamycin). The results of six independent experiments are shown.

**Figure 6. CD4$^+$CD25$^+$ T cells cultured in the presence of rapamycin, but not CsA, are able to inhibit ongoing T-cell responses.**

2.5$\times$10$^4$ freshly isolated CD4$^+$CD25$^+$ T cells were stimulated with 1$\times$10$^5$ $\gamma$-irradiated (30 Gy) HLA mismatched stimulator PBMC 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 co-culture assays. $^{ExpT_{REG}}$ derived from control (untreated), CsA-treated and rapamycin-treated cultures (X-axis) were co-cultured from day 0 (T=0) or day 2 (T=2) of MLR at a suppressor:effector ratio of 1:16 using 5$\times$10$^4$ responder CD4$^+$CD25$^-$ effector T-cells and 5$\times$10$^4$ $\gamma$-irradiated (30 Gy) stimulator PBMC. Proliferation at day 5 of culture was measured by $^3$H-thymidine incorporation, and is depicted as percentage of inhibition on the Y-axis. A representative experiment is shown. *$P<0.05$ for difference between groups.
Figure 1
Figure 2

Control

CsA
100 ng/ml  200 ng/ml  400 ng/ml

Rapamycin
25 nM  50 nM  100 nM
**Figure 3**

**A**

Stimulator CD4+CD25+ PBMC + Treg

- No drug
- CsA (400 ng/ml)
- Rapamycin (100 nM)

Culture For 7 days

Rest 2 days

Examine in suppression assay

**B**

![Graph showing 3H-incorporation](image)

- ExpTREG
- ExpTREG CsA
- ExpTREG Rapamycin

**C**

Suppressor : effector ratio

- 0 : 1
- 1 : 128

![Graph showing 3H-incorporation](image)
Figure 5

![Graph showing % Suppression vs. CD27+ TREG : CD27- TREG Ratio (Log scale)](image_url)
Figure 6

[Bar graph showing inhibition percentages for different conditions: ExpTREG, ExpTREG CsA, ExpTREG Rapamycin. Bars are labeled with MLR T=0 and MLR T=2. Symbols indicate statistical significance with * for MLR T=2.]
Rapamycin, and not Cyclosporin A, preserves the highly suppressive CD27+ subset of human CD4+CD25+ regulatory T cells

Jeroen J Coenen, Hans J Koenen, Esther v Rijssen, Luuk B Hilbrands and Irma Joosten