# Stability of human rapamycin-expanded CD4$^+$CD25$^+$ T regulatory cells

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*The online version of this article has a Supplementary Appendix.*

## ABSTRACT

### Background
The clinical use of *ex vivo*-expanded T-regulatory cells for the treatment of T-cell-mediated diseases has gained increasing momentum. However, the recent demonstration that FOXP3$^+$ T-regulatory cells may contain interleukin-17–producing cells and that they can convert into effector cells once transferred *in vivo* raises significant doubts about their safety. We previously showed that rapamycin permits the *ex vivo* expansion of FOXP3$^+$ T-regulatory cells while impairing the proliferation of non-T-regulatory cells. Here we investigated the Th17-cell content and the *in vivo* stability of rapamycin-expanded T-regulatory cells as pertinent aspects of cell-based therapy.

### Design and Methods
T-regulatory-enriched cells were isolated from healthy volunteers and were expanded *ex vivo* with rapamycin with a pre-clinical applicable protocol. T-regulatory cells cultured with and without rapamycin were compared for their regulatory activity, content of pro-inflammatory cells and stability.

### Results
We found that CD4$^+$CCR6$^+$CD161$^+$ T cells (*i.e.*, precursor/committed Th17 cells) contaminate the T-regulatory cells cultured *ex vivo* in the absence of rapamycin. In addition, Th17 cells do not expand when rapamycin-treated T-regulatory cells are exposed to a "Th17-favorable" environment. Rapamycin-expanded T-regulatory cells maintain their *in vitro* regulatory phenotype even after *in vivo* transfer into immunodeficient NOD-SCID mice despite being exposed to the irradiation-induced pro-inflammatory environment. Importantly, no additional rapamycin treatment, either *in vitro* or *in vivo*, is required to keep their phenotype fixed.

### Conclusions
These data demonstrate that rapamycin secures *ex vivo*-expanded human T-regulatory cells and provide additional justification for their clinical use in future cell therapy-based trials.

Key words: T-regulatory cells, *ex vivo* expansion, rapamycin, cell therapy, T-regulatory-cell stability.

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## Introduction

In recent years, there has been growing recognition of the ability of human FOXP3+ T-regulatory (Treg) cells to suppress adverse immune responses. Much research effort has been devoted to implementing Treg-cell therapy in the clinic[1,2] and results of the first clinical trials have been recently reported.[3,4] Given their reduced circulating frequency (*i.e.*, 5-10% of the CD4+ T cells) FOXP3+ Treg cells, to be of any therapeutic use in cell-therapy trials, need to be expanded *ex vivo*. The intrinsic reduced ability of Treg cells to proliferate *in vitro* can be reversed by potent stimulation through the T-cell receptor (TCR) in the presence of high doses of exogenous interleukin (IL)-2.[5] Protocols for the expansion of human FOXP3+ Treg cells performed under conditions of good manufacturing practice (GMP) have been shown to be feasible.[3,6-8] Importantly, these culture conditions are also highly advantageous for the expansion of effector CD25+ T cells, which may contaminate the starting peripheral FOXP3+ Treg cells. This obstacle can be bypassed by: (i) the use of cord blood Treg cells,[4] (ii) isolation of very pure peripheral Treg cells through flow-based cell sorters[3,9,10] or (iii) the addition of rapamycin to the culture, which preferentially inhibits proliferation of effector T cells while sparing Treg cells.[11,12]

IL-17 is a pro-inflammatory cytokine with non-redundant functions in the clearance of extracellular pathogens.[13] In humans, IL-17 is associated with many inflammatory disorders such as rheumatoid arthritis, asthma, multiple sclerosis, Crohn's disease and allograft rejection.[14] The majority of IL-17 derives from a population of CD4+ T cells known as Th17.[13] The combination of cytokines which stimulates the development and stabilizes human Th17 cells is a subject of much debate.[15] It appears that transforming growth factor (TGF)-β, IL-21, IL-23, IL-6, and IL-1β at various dosages and combinations, depending on the experimental conditions and the starting T-cell subset, are essential for Th17-cell generation/expansion.[15]

It has recently been shown that a memory fraction of CD25highFOXP3+ T cells contains a significant number of IL-17-producing cells which lack regulatory activity.[16,17] In the context of concerted efforts to use expanded populations of Treg cells for adoptive therapy in human T-cell-mediated diseases, the risk of Th17-cell contamination is a key, as transfer of contaminant pro-inflammatory cells may exacerbate rather than ameliorate disease. Thus, developing methods of securing *ex vivo*-expanded Treg cells intended for clinical use and inhibiting their pro-inflammatory potential *in vivo* have become all-important aspects of Treg cell-mediated immunotherapy.[18]

We set up a clinically applicable protocol for the expansion of human peripheral Treg cells using rapamycin to set the basis for an investigator-initiated clinical trial. Th17-cell content and the *in vivo* stability of the rapamycin-expanded human FOXP3+ Treg cells were investigated as critical pertinent aspects of cell-based therapy.

## Design and Methods

### Blood samples

Peripheral blood was obtained from healthy donors' buffy coats obtained after written informed consent in accordance with the Declaration of Helsinki under the protocol approved by the San Raffaele Scientific Institute Ethics Committee (protocol TIGET PERIBLOOD).

### T-regulatory cell purification

Peripheral blood mononuclear cells (PBMC) were separated by density-gradient centrifugation over Lymphoprep (Axis-shield, Oslo, Norway). Some PBMC were cryopreserved for later use. The remaining PBMC were processed using CD8 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Treg cells were then isolated from the negative cell fractions (CD8- cells) using CD25 MicroBeads (Miltenyi Biotec) following the manufacturer's instructions.

### Flow cytometry

The cells were stained for surface antigens with the following monoclonal antibodies: anti-CD4 PerCP (clone SK3, BD, Franklin Lakes, NJ, USA), anti-CD25 APC (clone 2A3, BD), CD161 PE (clone HP3G10, eBioscience), and CCR6-biotin (clone 11A9, BD) and streptavidin APC7 (BD). Intracellular staining for human FOXP3 and Helios was performed using the anti FOXP3-Alexa 488 and anti-Helios-PE monoclonal antibodies (clone 259D and clone 22F6, respectively, BioLegend, San Diego, CA, USA) according to the manufacturer's instructions. Samples were acquired on a FACSCanto (BD) and analyzed with FCS Express V3 software (DE Novo Software, Los Angeles, CA, USA).

### Cell cultures

CD8-CD25+ T cells isolated from PBMC of healthy subjects were plated at $0.1\times10^6$/mL in X-vivo 15 medium (Lonza, Verviers, Belgium) supplemented with 5% pooled AB human serum (Lonza) and 100 U/mL penicillin/streptomycin (Bristol-Myers Squibb, Sermoneta, Italy) in the presence or absence of 100 nM rapamycin (i.e. 100 ng/mL) (Rapamune®, Whyet, Maidenhead, Berkshire, UK).[12] The cells were activated with GMP grade beads coated with anti-CD3 and anti-CD28 antibodies (T Cell Expander® from Dynal Biotech ASA, Oslo, Norway) at a 1:3 ratio (cells:beads). At day 2 post-activation, IL-2 (500 U/mL) (Proleukin®, Chiron, Amsterdam, the Netherlands) was added to the culture and replenished as shown in Figure 1. Beads were removed after 14 days of culture by magnetic adherence and the expanded cells were used for further analyses.

### Suppression assay with $T_{MED}$ and $T_{RPM}$ cells

Previously frozen PBMC were thawed and stained with CFSE (Molecular Probes, Eugene, OR, USA)[11] and a suppression assay was performed as described elsewhere by our group.[12] The percentage of CFSE+ divided cells was calculated using the formula: [(n. of precursors that proliferated$_{1-6}$/n. of total precursors$_{0-6}$)x100]. The percentage of CFSE+ cells divided in the presence of expanded cells was compared to the percentage of CFSE+ divided cells in the absence of any added cells.[12]

### Cytokine detection in $T_{MED}$ and $T_{RPM}$ cells

The 14-day cultured T cells were plated at a density of $0.5\times10^6$ cells in 500 μL in 48-well plates and stimulated for 6 h with latex-beads (Molecular Probes) pre-coated with anti-CD3 (OKT3 clone) and anti-CD28 (clone CD28.2, BD Pharmingen) monoclonal antibodies at 500 ng and 50 ng for $10^6$ beads, respectively at a 1:2 ratio (cells:beads) in the presence of 10 μg/mL brefeldin A (Sigma-Aldrich). At the end of the 6-h activation, the cell surface was stained with anti-CD4 PerCp monoclonal antibody (clone SK3, BD). Subsequently, intracellular staining was performed with the following monoclonal antibodies: anti-IL-2 PE (clone MQ1-17H12), anti-IL-4 PE (clone MP4-25D2, anti-IL-10 PE (clone JES3-

9D7), anti-IFNγ APC (clone B27), and anti-TNFα PE (clone MaB11) (all from BD Pharmingen), and anti-IL-17 PE (clone eBio64CAP17, from eBioscence). Each stain was performed in the presence of anti-FOXP3 Alexa488 monoclonal antibody (clone 259D, Biolegend) according to the manufacturer's protocol.

### $T_H17$-cell differentiation/expansion

The $T_{MED}$ and $T_{RPM}$ cells were plated at a density of $0.5\times10^6$/mL and cultured with 5 ng/mL TGF-β (R&D System, Minneapolis, MN, USA), 25 ng/mL IL-21 (Cell Sciences, Canton, MA, USA) and 25 ng/mL IL-23 (R&D System) as previously described.[19] On day 3, IL-23 was replenished. On day 7 the cells (*i.e.*, $T_{MED}$-17, and $T_{RPM}$-17) were collected and used for further analysis.

### Suppression assay with $T_{MED}$-17 and $T_{RPM}$-17 cells

Previously frozen PBMC were thawed and stained with CFSE (Molecular Probes) as previously described.[11] $T_{MED}$-17 and $T_{RPM}$-17 cells were added at the ratio of 0.3:1 (T cells:responder cells) and stimulated with Treg Suppression Inspector beads (Miltenyi Biotec) at a ratio of 1:0.3 (cells:beads). Seven days later the cells were collected and stained with anti-CD4 and anti-CD8 monoclonal antibodies and analyzed by flow activated cell sorting (FACS).

### Cytokine detection in $T_{MED}$-17, $T_{RPM}$-17 cells

$T_{MED}$-17, $T_{RPM}$-17 cells were stimulated for 6 h with TPA (CalBiochem, Lucerne, Switzerland) and ionomycin (Sigma-Aldrich) at 10 ng/mL and 500 ng/mL, respectively, in the presence of 10 μg/mL brefeldin A (Sigma-Aldrich). At the end of the 6-h activation, the cell surface was stained with anti-CD4 and anti-CD25 monoclonal antibodies, while intracellular staining was performed with anti-IL-17 or anti-IL-2, and anti-IFNγ APC monoclonal antibodies.

### Non-obese diabetic/severe combined immunodeficiency mouse model

Six- to 8-week old female NOD/scid mice were obtained from Charles-River Italia (Calco, Italy). The experimental protocol was approved by the San Raffaele Scientific Animal Care and Use Committee (IACUC#387). The day before human cell transfer, mice were treated with 1 mg of anti-CD122 (TMβ-1) monoclonal antibody (Santa Cruz, Tebubio, Le Perray-en-Yuelines, France) i.p. as previously described.[20] On day 0, the mice received total body irradiation (TBI) with a single dose of 350 cGy (γ irradiation from a linear accelerator) and $8\times10^6$ cells/mouse were transferred i.p.. Three times a week the general appearance, body weight and mobility of the mice were evaluated and once a week peripheral blood was collected from the tail vein and chimerism analysis was performed. Human chimerism was determined weekly by flow cytometry, after bleeding from the tail vein, using the monoclonal antibodies anti-human-CD45 APC (clone HI30) and anti-mouse-CD45 PerCp (clone 30F11) (both from BD-Pharmingen). The percentage of chimerism was calculated using the formula: [h-CD45/(h-CD45+m-CD45)x100] as described elsewhere.[21] To improve human chimerism, some of the mice were treated with $2\times10^4$ IU/mouse/day i.p. of recombinant human IL2 (Proleukin®, Chiron) for 7 days after cell transfer.[21]

### Phenotype, cytokine production profile and suppressive activity of T cells recovered from non-obese diabetic/severe combined immunodeficiency mice

Thirteen or 19 days after human cell transfer into NOD/scid mice, the animals were sacrificed and peripheral blood, thymus, spleen and lymph nodes were collected and pooled after smashing ($T_{MED}$-mouse and $T_{RPM}$-mouse). The total cells recovered from each mouse varied accordingly to the cell-type injected. Total cells recovered from mice injected with $CD3^+$ T cells: $4.5\pm1.2\times10^6$, with $T_{MED}$: $0.2\pm0.5\times10^6$, and with $T_{RPM}$: $0.5\pm0.1\times10^6$ (average ± SD, n=7). Total cells from mice injected with $CD25^+T_{RPM}$ cells were analyzed by flow cytometry or were stimulated for 6 h with TPA (CalBiochem) and ionomycin (Sigma-Aldrich) in the presence of brefeldin A (Sigma-Aldrich). At the end of the 6-h activation, the cell surface was stained with anti-human CD4 PerCp (clone SK3, BD) and anti-human-CD45 APC (clone HI30, BD-Pharmingen) monoclonal antibodies. Subsequently, the intracellular staining was performed with anti-IL-17 or anti-IL-2, and anti-IFNγ monoclonal antibodies.

Alternatively, T cells were purified by using human-CD3 MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions and used as suppressor T cells in a suppression assay. Previously frozen PBMC (responder cells) were thawed and stained with CFSE (Molecular Probes) as described elsewhere.[11]

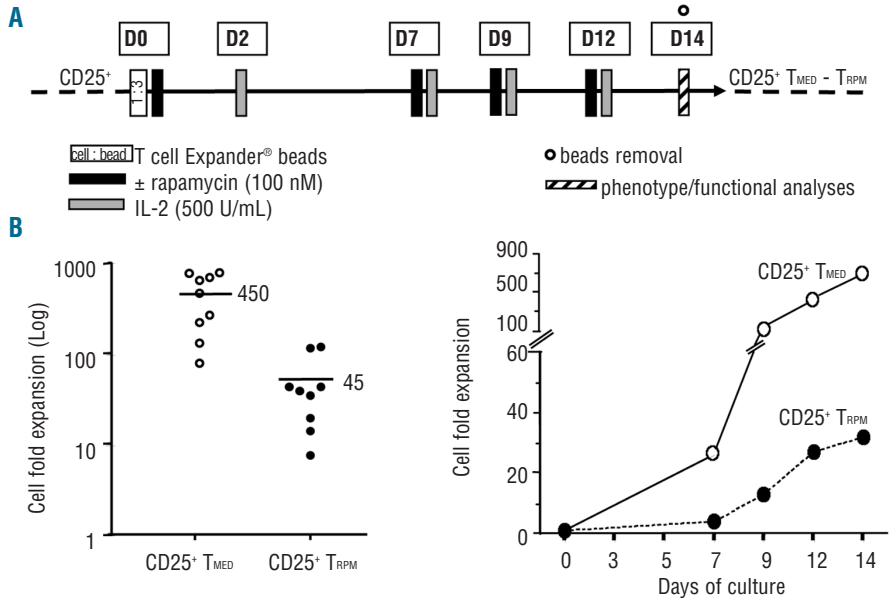



**Figure 1.** Protocol used for the expansion of enriched human $CD25^+$ T cells. (A) The diagram outlines the time points (D: day) and the procedures followed for the *ex vivo* expansion of $CD25^+$-enriched Treg cells. $T_{MED}$ are the cells expanded in the absence of rapamycin, $T_{RPM}$ are the cells expanded in the presence of rapamycin. The protocol is described in detailed in the *Design and Methods* section. (B) The total cell fold expansion after 14 days of culture was determined (left panel). $CD25^+$ $T_{MED}$ (empty symbols) and $CD25^+$ $T_{RPM}$ cells (filled symbols) were collected and counted at the end of the culture. Each dot represents one experiment. The average fold expansion is shown (bar and number). One representative time-course expansion of $CD25^+$ $T_{MED}$ (empty symbols) and $CD25^+$ $T_{RPM}$ cells (filled symbols) is presented (right panel).

$T_{MED}$-mouse and $T_{RPM}$-mouse cells were added at the ratio of 0.3:1 and 1:1 (T cells:responder cells) and stimulated with Treg Suppression Inspector beads (Miltenyi Biotec) at a ratio of 1:0.3 (cells:beads). Seven days later the cells were collected and stained with anti-CD4 and anti-CD8 monoclonal antibodies and analyzed by FACS.

### Statistical analysis

All statistical analyses were performed using a two-tailed Student's t-test. *P* values less than 0.05 were deemed statistically significant.

## Results

Human Treg cells were enriched from PBMC using GMP-grade available magnetic beads, which are the best alternative to clinical grade flow-based cell sorters.[8] We first depleted $CD8^+$ T cells and then positively selected $CD25^+$ cells. The purified cell subset (termed $CD25^+$) contained a median of 80±10% $CD4^+CD25^+$ cells and 61±5% of $CD25^+FOXP3^+$ cells (*data not shown*). $CD25^+$ cells were cultured *ex vivo* to obtain the best Treg-cell expansion in the shortest time possible. Increasing doses of GMP-grade anti-CD3/CD28–coated beads (from 1:1 to 1:5 cells:beads) and IL-2 (from 100 to 1000 U/mL), and different periods of culture (from 7 to 21 days) in the presence or absence of rapamycin ($T_{RPM}$ and $T_{MED}$ cells, respectively) were tested. The optimal expansion protocol was 14 days of culture in the presence of three beads per cell and 500 U/mL of exogenous IL-2 (scheme depicted in Figure 1A).

$T_{RPM}$ cells expanded 1 log less than $T_{MED}$ cells with a 7-day time-lag (Figure 1B), as we previously observed upon expansion of unfractionated human $CD4^+$ T cells.[12] $T_{RPM}$ cells contained an average of 51% FOXP3-expressing cells (range: 32-80%). Helios, a member of the Ikaros transcription factor family, is thought to be a specific marker of thymic-derived Treg cells.[22] We found an average of 73% (range, 60-85%) of $Helios^+$ cells within the FOXP3-expressing $T_{RPM}$ cells, suggesting a selective expansion of thymic-derived Treg cells in the presence of rapamycin. In addition, $T_{RPM}$ cells displayed a reproducible *in vitro* suppressive capacity towards both autologous and allogeneic responder T cells in a dose-dependent manner. In contrast, $CD25^+$ T cells expanded in the absence of rapamycin (*i.e.*, $T_{MED}$ cells) almost completely lost FOXP3-expressing cells (average: 14%; range, 3-30%) and the few cells remaining $FOXP3^+$ expressed low levels of Helios (average: 28%; range, 20-35%) and retained no *in vitro* suppressive function in any condition tested (Figure 2 and *Online Supplementary Figure S1*). The TCR Vβ repertoire remained polyclonal in both $T_{MED}$ and $T_{RPM}$ cells demonstrating the absence of a specific T-cell clone expansion in the 14-day culture (*Online Supplementary Table S1*). We previously showed that rapamycin can be considered as a "selective Treg cell growth-survival" factor since it inhibits proliferation of non-Treg cells,[12] which follow different molecular signaling pathways than Treg cells.[23,24] Analysis of the cytokine production profile of $T_{MED}$ and $T_{RPM}$ cells confirmed that the presence of rapamycin in the culture reduced the frequency of T cells releasing pro-inflammatory cytokines (Figure 3).

Recent findings demonstrate that $FOXP3^+$ T cells contain some IL-17-producing non-Treg cells[16,17] and that naïve $FOXP3^+$ Treg cells can differentiate into Th17 cells in the presence of IL-2 and lineage-specific polarizing factors.[25] As a consequence, we wondered whether the $CD25^+$ T-cell starting population contained significant numbers of Th17-cells or Th17-cell precursors, recently found to be highly enriched within the $CCR6^+CD161^+$ cell fraction,[26,27] which might expand upon the 14-day culture. The starting $CD25^+$

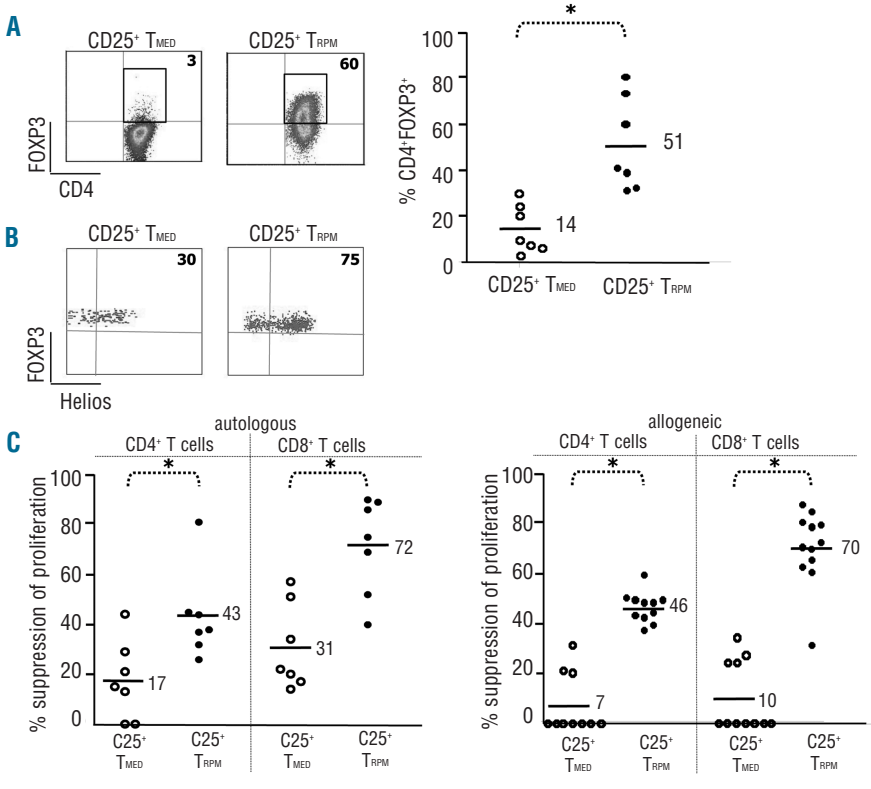



**Figure 2.** Phenotype and suppressive function of 14-day cultured $CD25^+$ T cells. (A) The frequency of $CD4^+FOXP3^+$ T cells within $CD25^+$ $T_{MED}$ and $CD25^+$ $T_{RPM}$ cells was established by FACS by gating on alive cells. One representative dot plot is shown on the left and all the results are included in the right vertical scatter plot in which each dot represents one experiment. The average $CD4^+FOXP3^+$ T-cell content is shown (bar and number). (B) The frequency of $Helios^+$ cells within $CD25^+$ $T_{MED}$ and $CD25^+$ $T_{RPM}$ cells was established by FACS by gating on $CD4^+FOXP3^+$ T cells (as shown in panel A). One representative dot plot from three experiments is shown. (C) The suppressive ability of $CD25^+$ $T_{MED}$ (empty symbols) and $CD25^+$ $T_{RPM}$ cells (filled symbols) was tested by co-culture experiments with CSFE-labeled autologous (left scatter plot) and allogeneic (right scatter plot) PBMC (responder cells) at a ratio of 0.4:1 (expanded T cells: responder cells). The amount of $CD4^+$ and $CD8^+$ $CFSE^+$ T cells proliferating in the absence or presence of cultured T cells was calculated as described in the *Design and Methods* section. Percentage of suppressive activity of each tested expanded T-cell population and the average suppressive function an shown (bar and number). Differences that are statistically significant are highlighted by an asterisk (*).

T-cell subset was slightly enriched in CCR6$^+$CD161$^+$ T cells, as compared to the unfractionated PBMC (Figure 4A). We, therefore, tested the IL-17-producing cell content in $T_{MED}$ and $T_{RPM}$ cells. Low amounts of IL-17$^+$IFN-γ$^-$ cells were present in the expanded CD25$^+$ T cells irrespectively of the presence of rapamycin (Figure 4B). Nevertheless, $T_{MED}$ cells contained a higher frequency of CCR6$^+$CD161$^+$ cells as compared to $T_{RPM}$ cells (Figure 4C). Rapamycin, therefore, inhibits the differentiation and growth of CCR6$^+$CD161$^+$ T cells during *ex vivo* expansion of CD25$^+$-enriched T cells. In spite of this, we could not rule out that a subsequent exposure of $T_{RPM}$ cells to a "Th17-favorable environment" in the absence of exogenous rapamycin would lead to Th17-cell differentiation and/or expansion. To resolve this issue, $T_{MED}$ and $T_{RPM}$ cells were cultured in the presence of IL-23, IL-21 and TGF-β (Figure 4D) (termed $T_{MED}$-17 and $T_{RPM}$-17), a cytokine-cocktail known to expand and stabilize Th17 cells.[13,19] Seven days of *in vitro* culture in this "Th17-favorable environment" and in the absence of rapamycin led to the expansion of IL-17-producing cells (at high or low frequency depending on the donor) in $T_{MED}$ but not in $T_{RPM}$ cells (Figure 4E). These data were also confirmed by a high frequency of CCR6$^+$CD161$^+$ cells in $T_{MED}$-17 but not in $T_{RPM}$-17 cells (*data not shown*). In addition, the FOXP3$^+$ cell-content (Figure 5A) and regulatory activity of $T_{RPM}$-17 cells were as good as those prior to the Th17-cell condition exposure (Figure 5B).

One major concern regarding the use of *ex vivo*-expanded Treg cells in immunotherapy trials is the risk of their *in vivo* conversion into effector cells and loss of suppressive ability. We, therefore, decided to test the stability of $T_{RPM}$ cells *in vivo* upon transfer into NOD/scid mice (protocol depicted in Figure 6A). $T_{MED}$, $T_{RPM}$, or freshly isolated CD3$^+$ T cells were injected into total body irradiated (TBI)-NOD/scid mice in the absence of any additional rapamycin treatment. T cells previously cultured *ex vivo* (*i.e.*, $T_{MED}$ and $T_{RPM}$ cells) did not expand *in vivo* as efficiently as freshly isolated T cells (Figure 6B), despite the fact that the frequency of circulating human CD3$^+$CD25$^+$ cells was almost identical in the mice receiving either freshly isolated T cells or $T_{MED}$ and $T_{RPM}$ cells 13 days following *in vivo* transfer (Figure 6C). This is likely due to T-cell exhaustion given by the powerful *ex vivo* TCR-mediated expansion, as previously demonstrated by others.[28] Additional treatment of recipient mice with recombinant human IL-2 did not improve human T-cell chimerism in any of the treated mice (*data not shown*). Despite the lack of evident circulation of human T cells in the peripheral blood of NOD/scid mice injected with $T_{MED}$ and $T_{RPM}$ cells, human cells were found in their spleens and lymph nodes. We, therefore, isolated human T cells from secondary lymphoid organs of NOD/scid mice 19 days after adoptive cell transfer. Interestingly, the recovered $T_{RPM}$ (*i.e.*, $T_{RPM}$-MOUSE) cells remained FOXP3$^+$ and almost all the FOXP3$^+$ cells were Helios–expressing cells and contained no CCR6$^+$CD161$^+$ precursor/committed Th17 cells (Figure 6D). The cytokine production profile of $T_{RPM}$-MOUSE cells changed slightly towards more IL-2 and IFN-γ producing cells (average 11% and 8%, respectively). Conversely, IL-17$^+$ cells were almost undetectable in all recovered $T_{RPM}$-MOUSE cells (Figure 6E). Unfortunately, the number of $T_{MED}$ cells recovered from the injected mice was too low to perform the above-mentioned tests. Importantly, $T_{RPM}$ cells retained their suppressive activity even 19 days after *in vivo* "parking" in TBI-treated host mice (Figure 6F) while neither unexpanded T cells nor $T_{MED}$ cells recovered from injected mice displayed any suppressive activity (*data not shown*).

Overall, these *in vitro* and *in vivo* data strongly suggest that rapamycin not only allows the *ex vivo* expansion of Treg cells voided of inflammatory-producing cells but also fixes

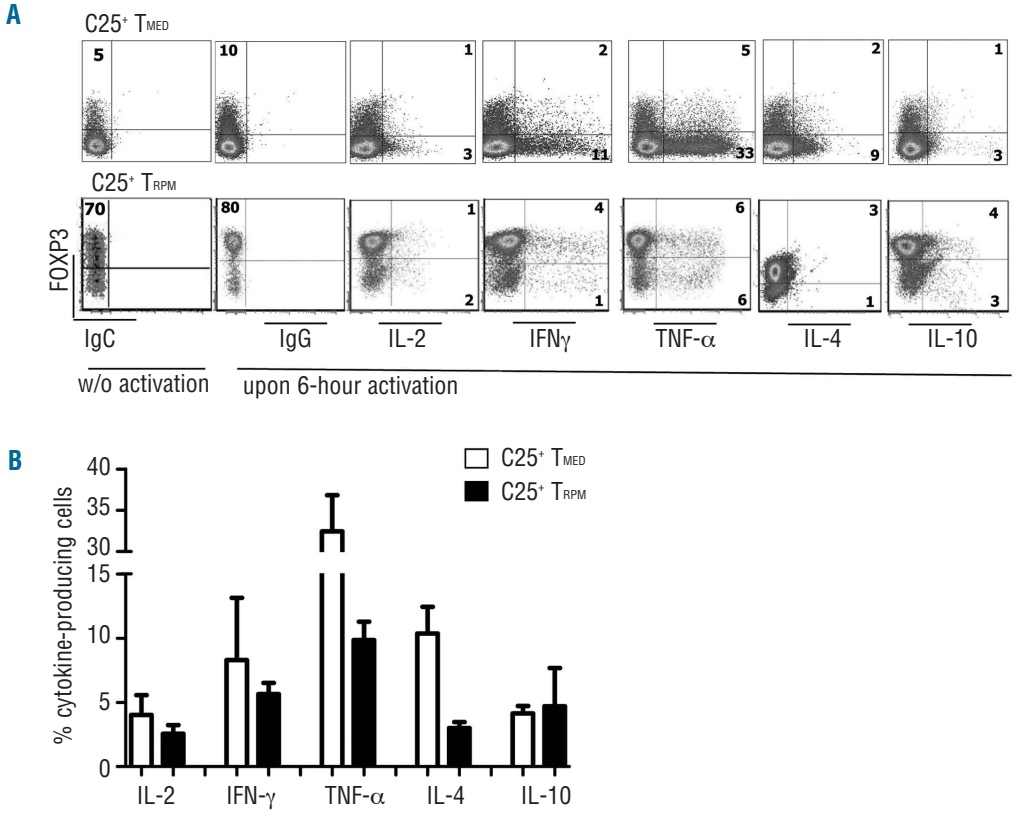



**Figure 3.** Cytokine production profile of 14-day cultured CD25$^+$ T cells. (A) The frequency of $T_{MED}$ and $T_{RPM}$ FOXP3$^{+/-}$ cells producing the indicated cytokines was established by FACS. The percentages of FOXP3$^+$ (upper right) and FOXP3$^-$ (lower right) cells producing the given cytokines upon 6-h activation in one representative experiment out of three are shown. FOXP3 expression prior and after 6-hour activation is shown. (B) The average cytokine production profile, irrespective of FOXP3 expression, upon 6-h activation of CD25$^+$ $T_{MED}$ (empty bars) and CD25$^+$ $T_{RPM}$ cells (filled bars) determined by FACS (n=3, mean ± SEM).

their phenotype.

## Discussion

Clinical trials with *ex vivo*-expanded human FOXP3$^+$ Treg cells isolated from peripheral blood are currently under question mainly because purified CD25$^+$ T cells may contain contaminant non-Treg cells.[16,17] The current study shows that a pre-clinical grade protocol for the expansion of functional peripheral Treg cells with rapamycin is feasible. In addition, we demonstrate that rapamycin-expanded human Treg cells are not contaminated by Th17 cells and that they maintain a stable phenotype even upon *in vivo* transfer with no need for additional rapamycin treatment.

We previously reported that rapamycin permits the preferential enrichment and subsequent expansion of mouse[11] and human[12] FOXP3$^+$ Treg cells from a population of unfractionated CD4$^+$ T cells. In these previous studies we showed that three rounds of powerful *in vitro* stimulation were required to expand an average of 30% FOXP3$^+$ cells with sustained suppressive activity. In this work we aimed to start with a purer Treg-cell fraction using GMP-grade available magnetic beads and to shorten the cell culture. Interestingly, the purity of our starting cell population was very similar to that recently reported by Brunstein *et al*. who have just performed a clinical trial in humans with *ex vivo* purified and expanded cord-blood derived CD25$^+$ T cells.[4] This further supports the validity of our approach for expanding clinical-grade peripheral CD25$^+$ T cells. In addition, it is clear that defining Treg cell dosing strategies in new clinical trials requires large-scale Treg-cell expansion capacity. The protocol described here led to an average 45-

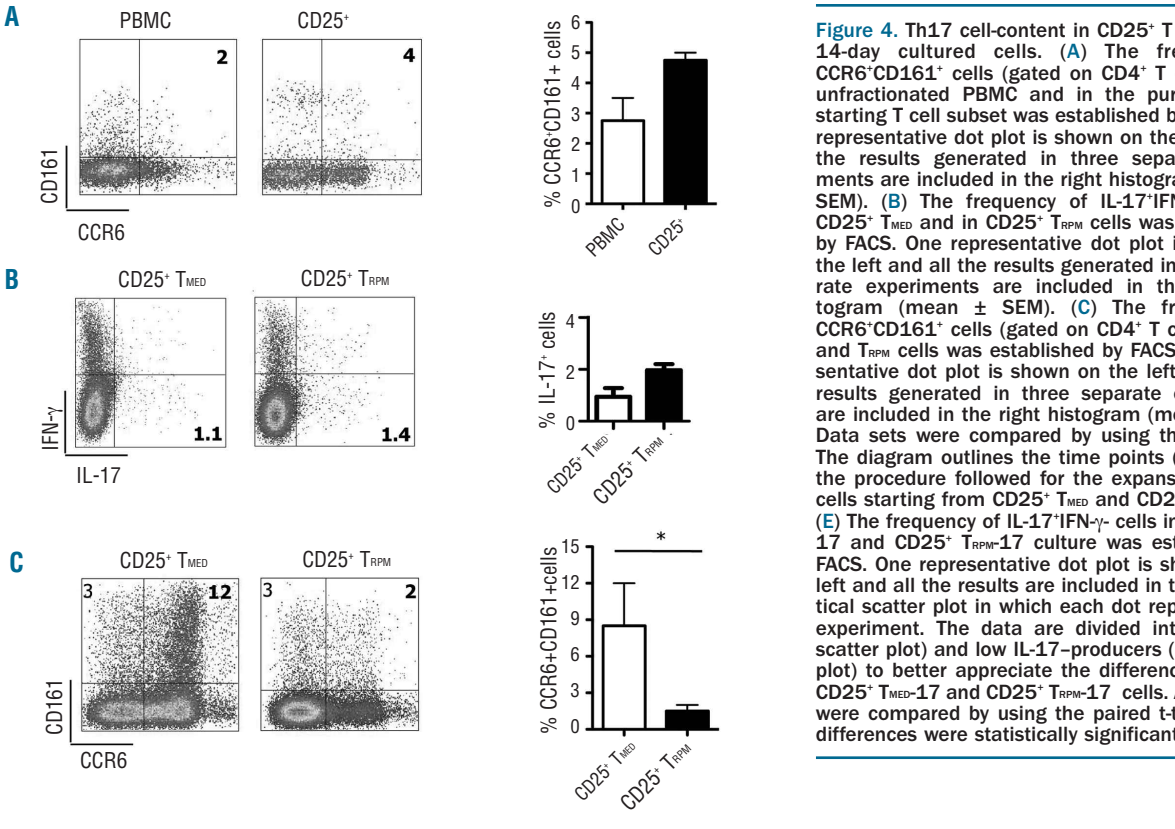



**Figure 4.** Th17 cell-content in CD25$^+$ T cells and in 14-day cultured cells. (A) The frequency of CCR6$^+$CD161$^+$ cells (gated on CD4$^+$ T cells) in the unfractionated PBMC and in the purified CD25$^+$ starting T cell subset was established by FACS. One representative dot plot is shown on the left and all the results generated in three separate experiments are included in the right histogram (mean ± SEM). (B) The frequency of IL-17$^+$IFN-γ$^-$ cells in CD25$^+$ $T_{MED}$ and in CD25$^+$ $T_{RPM}$ cells was established by FACS. One representative dot plot is shown on the left and all the results generated in three separate experiments are included in the right histogram (mean ± SEM). (C) The frequency of CCR6$^+$CD161$^+$ cells (gated on CD4$^+$ T cells ) in $T_{MED}$ and $T_{RPM}$ cells was established by FACS. One representative dot plot is shown on the left and all the results generated in three separate experiments are included in the right histogram (mean ± SEM). Data sets were compared by using the t-test. (D) The diagram outlines the time points (D: day) and the procedure followed for the expansion of Th17 cells starting from CD25$^+$ $T_{MED}$ and CD25$^+$ $T_{RPM}$ cells. (E) The frequency of IL-17$^+$IFN-γ- cells in CD25$^+$ $T_{MED}$-17 and CD25$^+$ $T_{RPM}$-17 culture was established by FACS. One representative dot plot is shown on the left and all the results are included in the right vertical scatter plot in which each dot represents one experiment. The data are divided into high (left scatter plot) and low IL-17–producers (right scatter plot) to better appreciate the differences between CD25$^+$ $T_{MED}$-17 and CD25$^+$ $T_{RPM}$-17 cells. All data sets were compared by using the paired t-test and the differences were statistically significant (*P*=0.036).

fold expansion of our starting Treg-cell fraction. This increase is limited as compared to that of other protocols performed in the absence of rapamycin[2] but is absolutely sufficient to obtain, for instance, $3\times10^6$/kg of expanded cells (for an average patient of 60 kg), which is the highest dose shown to be effective and safe in cord blood-transplanted patients.[4]

We believe that the novelty of our work lies in two major aspects. First, Th17 cells do not differentiate and expand upon *ex vivo* culture of magnetically-isolated CD25$^+$ Treg cells only in the presence of rapamycin. The recent findings demonstrating that memory FOXP3$^+$ T cells contain some IL-17-producing non-Treg cells[16,17] and that naïve FOXP3$^+$ Treg cells differentiate into Th17 cells in the presence of IL-2 and lineage-specific polarizing factors[25] raise significant doubts about the safety of the protocols commonly used for Treg-cell expansion. The experimental procedure must in fact prevent the growth and development of IL-17-producing or IL-17-precursor cells, which could contaminate the starting T-cell fraction. In our experimental conditions, the starting CD25$^+$ T-cell subset did indeed contain a certain number of already committed Th17 cells or their precursors[26,27] which expanded efficiently over the 14-day culture period only in the absence of rapamycin. Importantly, the subsequent exposure of $T_{RPM}$ cells to a Th17-favorable environment in the absence of new addition of rapamycin to the culture did not lead to Th17-cell differentiation or expansion. These data demonstrate that rapamycin imprints a fixed phenotype, not dependent upon the chronic presence of rapamycin, to the expanded Treg cells. The molecular mechanism underlying such a phenomenon is currently under active investigation.

It has been shown that rapamycin reduces *de novo, in vitro* generation of IL-17-producing cells by TGF-β+IL-6 while it permits TGF-β-induced development of FOXP3$^+$ cells starting from murine CD4$^+$ T cells.[29] This could be ascribed to blockade of the biological activity of IL-6 by rapamycin.[29] Our new findings demonstrate that rapamcyin has an inhibitory effect on human Th17-cell growth and development starting from a Treg-cell–enriched fraction and, importantly, it enables the expansion of Treg cells rich in Helios$^+$ cells and void of Th17-cell contaminants.

The second novel aspect of our work is the demonstration that $T_{RPM}$ cells preserve their regulatory function even upon *in vivo* transfer into TBI-treated immunodeficient mice in the absence of any additional rapamycin treatment, thus further supporting their fixed phenotype. The *in vivo* suppressive potential of FOXP3$^+$ Treg cells, freshly isolated[30] or previously expanded in the presence[31] or absence[32,33] of rapamycin, has already been shown in xeno-graft-*versus*-host disease (GvHD) murine models. However, to our knowledge, to date no experiments have been reported in which only *ex vivo*-expanded Treg cells have been transferred into TBI-treated NOD/scid mice and subsequently recovered. It is well known that pre-transplant irradiation of immunodeficient mice prompts a massive cytokine storm, which promotes human T-cell engraftment, activation and development of lethal xeno-GvHD.[34] The TBI-induced release of TNF-α, IL-1, and IL-6 promotes the activation of host antigen-presenting cells and up-regulation of MHC antigens,[35] which are crucial for the induction of lethal xeno-GvHD by human T cells.[36] We found that expanded Treg cells have a reduced *in vivo* proliferative capacity as compared to the same number of freshly isolated T cells, confirming data from the past performed with total T cells.[28] However, the transferred T cells did survive for as long as 19 days after *in vivo* transfer (latest time point analyzed) and despite being exposed to the critical TBI-induced cytokine storm their phenotype and suppressive activity remained stable.

It is evident that many issues need to be resolved prior to using *ex vivo*-expanded human peripheral FOXP3$^+$ Treg cells in clinical trials.[2] A common point of discussion among scientists with a strong interest in Treg-cell therapies is the limit of cytokine-producing expanded Treg cells that can be safely infused into a patient. Our data show that, upon *in vivo* transfer, Treg cells skew their cytokine production profile a bit more towards INF-γ/IL-2-producing cells but, importantly, this profile does not lead to a reduced regulatory function and the mere transfer of these cells *in vivo* does not cause xeno-GvHD. We, therefore, believe that rather than setting a "cytokine-producing Treg cell threshold" one

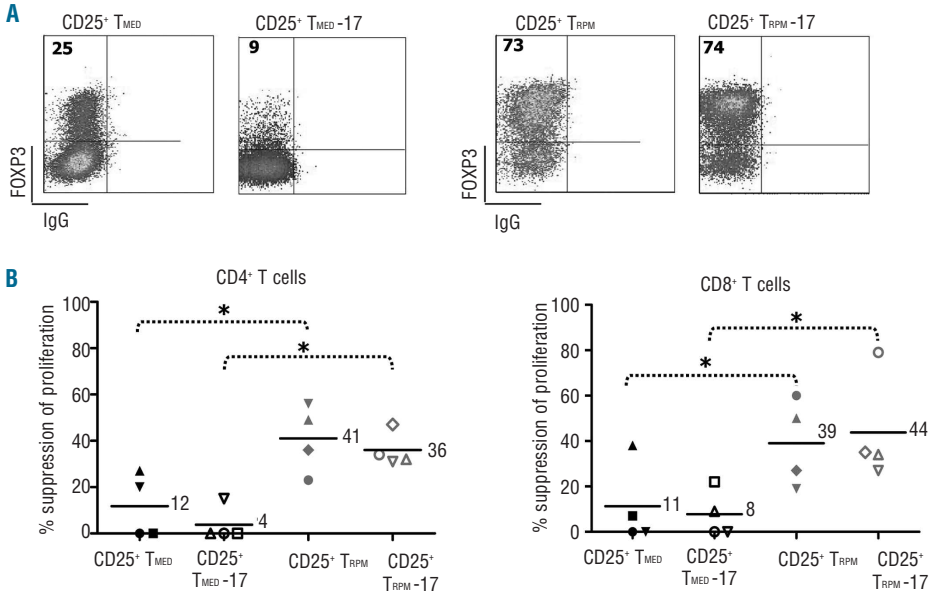
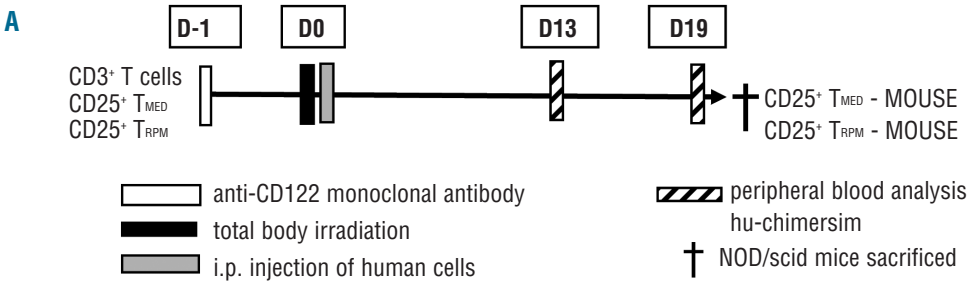
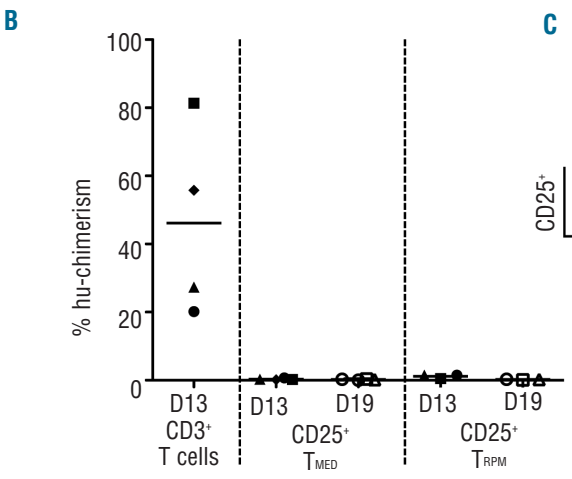



**Figure 5.** FOXP3 cell content and suppressive ability of CD25$^+$T-17 cells. (A) The frequency of FOXP3$^+$ T cells within CD25$^+$$T_{MED}$ and CD25$^+$$T_{RPM}$ prior and after Th17 cell expansion was established by FACS. One representative dot plot out of four independent experiments is shown. (B) The suppressive ability of CD25$^+$ $T_{MED}$, CD25$^+$ $T_{MED}$-17, CD25$^+$ $T_{RPM}$ cells and CD25$^+$ $T_{RPM}$-17 cells was tested by co-culture experiments with CSFE-labeled PBMC (responder cells) at a ratio of 0.3:1 (expanded T cells : responder cells). The amount of CD4$^+$ and CD8$^+$ CFSE$^+$ T cells proliferating in the absence or presence of cultured T cells was calculated as described in the *Design and Methods* section. Percentage of suppressive activity of each tested expanded T-cell populations and the average suppressive function are shown (bars and numbers). Differences that are statistically significant are highlighted by an asterisk (*).



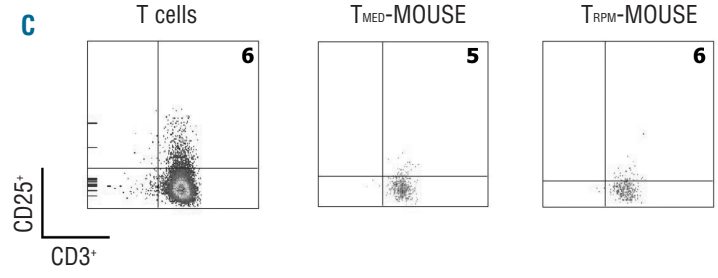
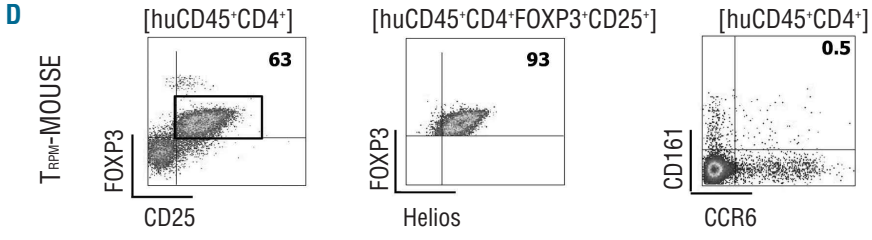
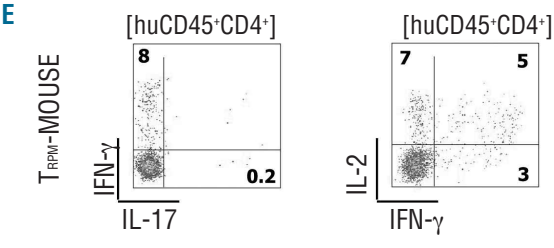




**Figure 6 A-C.** *In vivo* plasticity of 14-day cultured CD25+ T cells. (A) The diagram outlines the time points (D: day) and the procedure followed for the *in vivo* transfer into NOD/scid mice of freshly isolated human CD3+ T cells, or 14-day cultured CD25+ TMED and CD25+ TRPM. (B) Human chimerism detected in the peripheral blood of NOD/scid mice upon day 13 and day 19 of *in vivo* transfer of the indicated cells. (C) The frequency of circulating human CD3+CD25+ T cells was established by FACS by gating on human-CD45+ cells 13 days following *in vivo* transfer. One dot plot representative of one mouse out of three mice per group is shown.



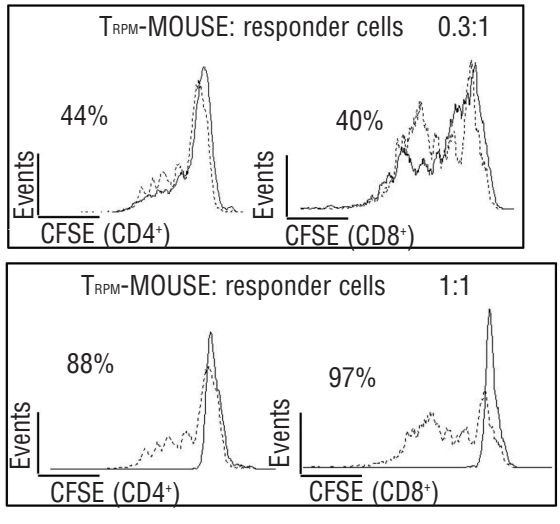




**Figure 6 D-F.** (D) The phenotype of CD25+TRPM-MOUSE cells recovered 19 days after transfer into NOD/scid mice was tested. The frequency of CD25+FOXP3+T cells was established by FACS by gating on hu-CD45+CD4+ cells (left dot plot); the frequency of Helios+ cells was established by FACS by gating on hu-CD45+CD4+FOXP3+CD25+ cells (middle dot plot); and the frequency of CD161+CCR6+ T cells was established by FACS by gating on hu-CD45+CD4+ cells (right dot plot). One dot plot representative of one mouse out of three mice analyzed is shown. (E) The cytokine production profile of CD25+TRPM-MOUSE cells recovered 19 days after transfer into NOD/scid mice was tested. The frequency of IL-17+ and IFN-γ+ T cells (left dot plot) and the frequency of IFN-γ+/IL-2+ T cells (right dot plot) was established by FACS by gating on human CD45+CD4+ cells. One dot plot representative of one mouse out of three mice tested is shown. (F) The suppressive ability of CD25+TRPM-MOUSE cells recovered 19 days after transfer into NOD/scid mice was tested. CSFE-labeled allogeneic PBMC (responder cells) were cultured with CD25+TRPM-MOUSE cells at a ratio of 0.3:1 (upper panel) and 1:1 (lower panel) (TRPM-MOUSE cells: responder cells). The amount of CFSE+ T cells proliferating in the absence (dotted lines) or presence (solid lines) of the given cells was calculated as described in the *Design and Methods* section. Percentages of suppressive activity of each tested T-cell population are shown. One representative experiment is shown.

should set up appropriate tests to monitor the regulatory stability of the transferred cells.

In summary, the demonstration that Treg-enriched cells expanded in the presence of rapamycin lack contaminant Th17 cells and are stable upon *in vivo* transfer in a murine model of TBI-induced inflammation in the absence of rapamycin administration provides additional justification for the clinical use of these cells in future cell therapy-based trials.

## Authorship and Disclosures

*The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.*

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