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Rapamycin Promotes Expansion of Functional $CD4^+CD25^+FOXP3^+$ Regulatory T Cells of Both Healthy Subjects and Type 1 Diabetic Patients[1]

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Rapamycin Promotes Expansion of Functional $CD4^+CD25^+FOXP3^+$ Regulatory T Cells of Both Healthy Subjects and Type 1 Diabetic Patients[1]

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$CD4^+CD25^+FOXP3^+$ T regulatory cells (Tregs) are pivotal for the induction and maintenance of peripheral tolerance in both mice and humans. Rapamycin has been shown to promote tolerance in experimental models and to favor $CD4^+CD25^+$ Treg-dependent suppression. We recently reported that rapamycin allows in vitro expansion of murine $CD4^+CD25^+FoxP3^+$ Tregs, which preserve their suppressive function. In the current study, we show that activation of human $CD4^+$ T cells from healthy subjects in the presence of rapamycin leads to growth of $CD4^+CD25^+FOXP3^+$ Tregs and to selective depletion of $CD4^+CD25^-$ T effector cells, which are highly sensitive to the antiproliferative effect of the compound. The rapamycin-expanded Tregs suppress proliferation of both syngeneic and allogeneic $CD4^+$ and $CD8^+$ T cells. Interestingly, rapamycin promotes expansion of functional $CD4^+CD25^+FOXP3^+$ Tregs also in type 1 diabetic patients, in whom a defect in freshly isolated $CD4^+CD25^+$ Tregs has been reported. The capacity of rapamycin to allow growth of functional $CD4^+CD25^+FOXP3^+$ Tregs, but also to deplete T effector cells, can be exploited for the design of novel and safe in vitro protocols for cellular immunotherapy in T cell-mediated diseases. *The Journal of Immunology,* **2006, 177: 8338–8347.**

In healthy individuals, multiple mechanisms of central immunological tolerance eliminate or inactivate lymphocytes that bear receptors specific for autoantigens in the thymus. Nevertheless, some autoreactive lymphocyte clones escape these mechanisms and are present within the peripheral lymphocyte pool. One way by which the pathogenic potential of autoreactive T cell clones is kept in check in the periphery is through T regulatory cells (Tregs)[3] (reviewed in Ref. 1). $CD4^+$ Tregs that constitutively coexpress the IL-2Rα chain ($CD4^+CD25^+$) have been shown to play a critical role in controlling undesired immune responses to self Ags. Seminal experiments were performed by Sakaguchi (reviewed in Ref. 2), who demonstrated that depletion of $CD4^+CD25^+$ Tregs results in systemic autoimmune diseases in mice. Furthermore, transfer of these Tregs prevented development of autoimmune diseases. Human $CD4^+CD25^+$ T cells with regulatory capacity have also been described (3–5). Human $CD4^+CD25^+$ Tregs, similarly to the murine counterpart, are generated in the thymus and are characterized by the ability to suppress proliferation of responder T cells through a cell-cell contact-dependent mechanism, the inability to produce IL-2, and the anergic phenotype in vitro. This hyporesponsiveness can be reversed by potent stimulation via TCR and high concentrations of IL-2 (reviewed in Ref. 6).

Human $CD4^+CD25^+$ T cells can be split into suppressive ($CD25^{high}$) and nonsuppressive ($CD25^{low}$) cells, according to the level of CD25 expression (7). However, analysis at the clonal level demonstrated that even the small fraction of $CD25^{high}$ cells is not a homogeneous population of suppressor T cells (8). A member of the forkhead family of transcription factors, FOXP3, has been shown to be expressed in murine and human $CD4^+CD25^+$ Tregs and appears to be a master gene controlling $CD4^+CD25^+$ Treg development (reviewed in Ref. 9). Recently, it has been demonstrated that the majority of $FOXP3^+$ T cells are $CD127^{low/-}$. Thus, selection with a broad gating strategy on $CD4^+CD25^{high}$ $CD127^{low/-}$ results in highly purified $FOXP3^+$ Tregs (10, 11).

It is now evident that Tregs are important in controlling human autoimmune diseases. $CD4^+CD25^+$ Tregs with a reduced in vitro suppressive function were found in some studies performed on patients with type 1 diabetes (T1D) (12, 13), multiple sclerosis (14), autoimmune polyglandular syndrome type II (15), and myasthenia gravis (16). In all cases, the mechanistic basis for this defect remains to be elucidated.

Given the defective function or low cell numbers of Tregs in patients with autoimmune diseases, replacement with $CD4^+CD25^+$ Tregs from normal donors (ND) or expansion of autologous functional $CD4^+CD25^+$ Tregs represents an interesting therapeutic approach. Because $CD4^+CD25^+$ Tregs represent only 3–5% of the peripheral blood, there is a need of reliable expansion protocols. However, in vitro expansion of $CD4^+CD25^+$ T cells from patients with ongoing T cell-mediated diseases has an intrinsic risk of also expanding activated $CD4^+$ T effector cells contaminating the $CD25^+$ T cell compartment. The design of efficient and safe protocols for the selective expansion of Tregs is therefore fundamental for their future clinical application.

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[3] Abbreviations used in this paper: Treg, T regulatory cell; GITR, glucocorticoid-induced TNF receptor; ND, normal donor; PI, propidium iodide; T1D, type 1 diabetes.

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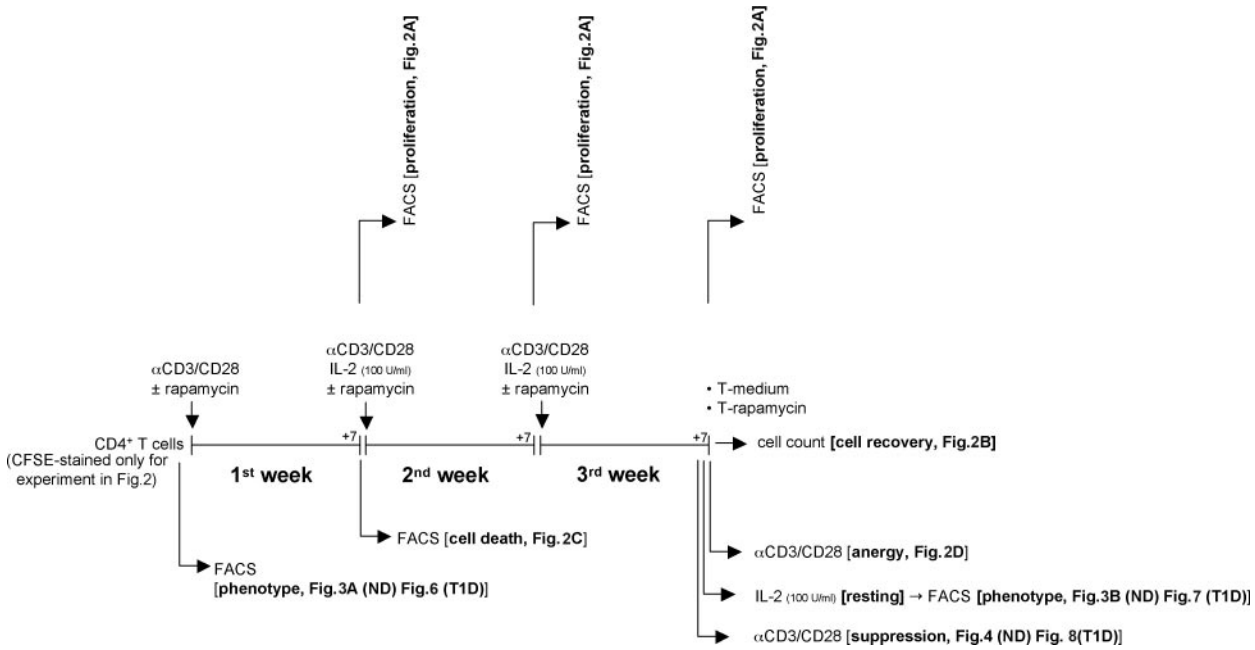

FIGURE 1. Detailed scheme of the experiments performed in the study.

We recently demonstrated that rapamycin, an immunosuppressive compound currently used to prevent acute graft rejection in humans (17), selectively expands the murine $CD4^+CD25^+$ $FoxP3^+$ Tregs in vitro. These expanded Tregs suppress proliferation of T cells in vitro and prevent allograft rejection in vivo (18). In the above mentioned study, the effect of rapamycin on human Tregs and the mechanisms responsible for the rapamycin-mediated Treg expansion were not explored. In this study, we now provide new evidence that in vitro activation of a bulk population of human $CD4^+$ T cells in the presence of rapamycin induces expansion of $CD4^+CD25^+FOXP3^+$ Tregs, which suppress both syngeneic and allogeneic $CD4^+$ and $CD8^+$ T cell proliferation. Rapamycin, by selectively blocking expansion and proliferation of $CD4^+CD25^-$ T effector cells, spares and promotes growth of $CD4^+CD25^+$ Tregs normally present in the circulation. Importantly, rapamycin selectively expands suppressive $CD4^+CD25^+FOXP3^+$ Tregs also from T1D patients, in whom a functional defect in freshly isolated $CD4^+CD25^+$ Tregs has been reported (12, 13).

Materials and Methods

Blood samples

Peripheral blood was obtained from healthy donors in accordance with local ethical committee approval. Peripheral blood from T1D patients (mean age, 19.15 ± 4.33 years; disease duration, 16.5 ± 7.6 mo), upon informed consent, was provided by A. Ziegler (Diabetes Research Institute, Munich, Germany).

Cell purification

PBMC were separated by density-gradient centrifugation over Lymphoprep (Amersham Biosciences). $CD4^+$ T cells were purified by negative selection using the untouched $CD4^+$ T cell isolation kit (Miltenyi Biotec), according to the manufacturer's instructions. A portion of the resultant $CD4^+$ T cells and all the $CD4^-$ cells were cryopreserved for later use. The average purity of $CD4^+$ T cells was 94.3 ± 3.9% ($n = 14$).

Flow cytometry

Cells were stained with the indicated surface Abs, all from BD Biosciences, except for glucocorticoid-induced TNF receptor (GITR) and CCR7 (R&D Systems) and were analyzed with a FACScan flow cytometer equipped with CellQuest software (BD Biosciences). Intracytoplasmic staining for human FOXP3 was performed using the anti-FOXP3 staining kit (eBioscience), according to the manufacturer's instructions. Of note is that the anti-human CD25 PE mAb (BD Biosciences) provides a brighter FACS signal than the anti-human CD25 Cy mAb (BD Biosciences).

T cell cultures

$CD4^+$ T cells isolated from PBMC of healthy subjects or T1D patients were activated with plate-bound anti-CD3 (OKT3 clone, 10 μg/ml) and soluble anti-CD28 (CD28.2 clone, 1 μg/ml) mAbs (BD Biosciences). Cells were cultured in the presence of X-vivo 15 medium (BioWhittaker) supplemented with 5% pooled AB human serum (BioWhittaker) and 100 U/ml penicillin/streptomycin (Bristol-Myers Squibb) in the presence or absence of 100 nM rapamycin (i.e., 1 μg/ml) (Sigma-Aldrich). Lower doses of rapamycin were also tested (namely 1 and 10 nM); however, a reproducible expansion of Tregs was observed only in the presence of 100 nM rapamycin (data not shown). Three rounds of stimulation of 7 days each were performed (Fig. 1). IL-2 (BD Biosciences) was added starting from the second round of stimulation at 100 U/ml.

Suppression experiments

Purified $CD4^+$ T cells or $CD4^-$ PBMC (average $CD8^+$ T cells content: 42 ± 14.1%; $n = 9$) from healthy subjects or T1D patients were stained with CFSE (Molecular Probes), as described elsewhere (18), and cultured in 96-well plates (2×10^5/well) precoated with anti-CD3 (10 μg/ml) and with soluble anti-CD28 (1 μg/ml) mAbs (BD Biosciences). T cells cultured for 3 wk in medium or rapamycin were first stained with SNARF (Molecular Probes) following the same protocol as for CFSE staining, and subsequently were added in 1:1 ratio (i.e., 10^5:10^5) to the culture. Seven days later, the cells were collected and analyzed by FACS. In case of responder $CD4^-$ T cells, the culture was stained with anti-CD8 Cy mAb. The proportion of $CFSE^+$ (FL-1)-responding T cells proliferating in vitro was calculated by gating on lymphocytes + alive cells ($TOPRO^-$ FL-4) (Molecular Probes) + $CD8^+$ T cells (FL-3) (in case of $CD4^-$ responder cells) and by excluding $SNARF^+$ (FL-2) cells. The number of gated cells (events) in a given cycle (division: n) was divided by 2 raised to power n, to calculate the percentage of original precursor cells from which they arose. The sum of original precursors from division 1 to 6 represents the number of precursor cells that proliferated. The percentage of $CFSE^+$ divided cells was calculated by ((number of precursors that proliferated$_{1-6}$/number of total precursors$_{0-6}$) × 100) (18). The percentage of $CFSE^+$ cells divided in the presence of cultured cells was compared with percentage of $CFSE^+$ divided cells in the absence of any added cells.

Statistical analysis

All statistical analyses were performed using the Student two-tailed t test, and values at $p < 0.05$ were deemed significant.

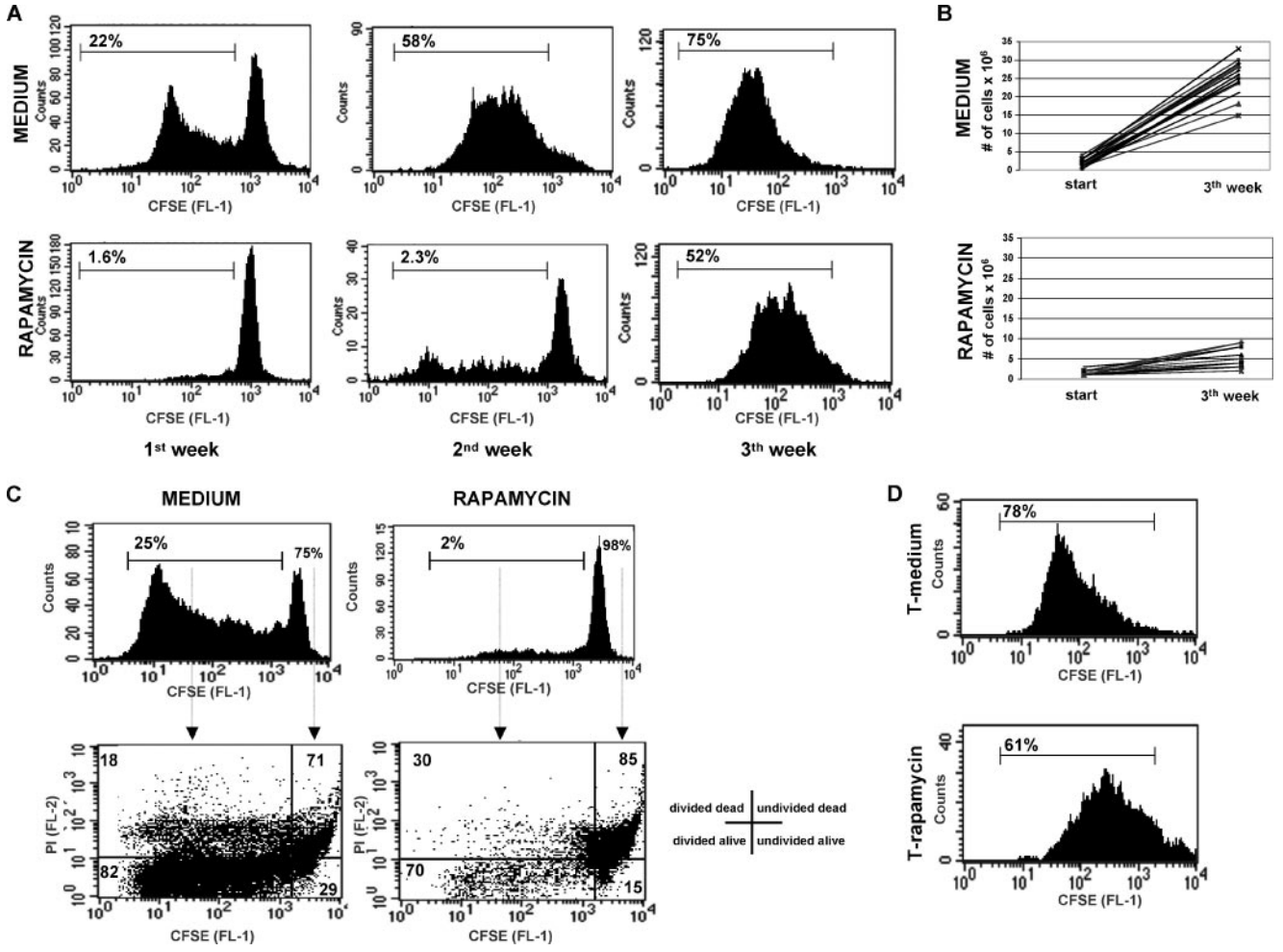

FIGURE 2. Rapamycin markedly reduces CD4$^+$ T cell proliferation, but does not induce T cell anergy. *A*, Peripheral CD4$^+$ T cells were stained with CFSE either at the beginning of the culture (first week), or before the second (second week) or the third (third week) round of stimulation. CFSE$^+$ CD4$^+$ T cells were activated with anti-CD3 + anti-CD28 mAbs in the presence or absence of 100 nM rapamycin. CFSE dilution was measured 7 days after activation. Numbers indicate the percentage of dividing cells. One representative experiment of six is presented. *B*, Number of CD4$^+$ T cells present at the beginning and at the end of three rounds of stimulation in medium or rapamycin is shown. Each line represents one experiment. There was a significantly higher amount of cells recovered in medium cultures than in rapamycin cultures ($p < 0.001$). *C*, CD4$^+$ T cells were stained with CFSE and were activated with anti-CD3 + anti-CD28 mAbs in the presence or absence of 100 nM rapamycin. After 7 days, cells were stained with PI and analyzed by FACS. Histograms show cell proliferation in the presence or absence of rapamycin, and numbers indicate the percentage of divided and undivided cells. Dot plots show CFSE dilution vs PI incorporation. The percentages of dead (PI$^+$) and alive cells (PI$^-$) within divided and undivided gated cells (indicated by the arrows) are indicated. One representative experiment of three is presented. *D*, After three rounds of stimulation in the absence (T-medium) or presence of rapamycin (T-rapamycin), the cells were stained with CFSE and restimulated with anti-CD3 + anti-CD28 mAbs in the absence of rapamycin and of exogenous IL-2. CFSE dilution was monitored 7 days after activation. Numbers indicate the percentage of dividing cells. One representative experiment of three is presented.

Results

Rapamycin markedly reduces CD4$^+$ T cell proliferation in vitro, but does not induce T cell anergy

To define the effect of rapamycin on human T cells, CD4$^+$ T cells purified from peripheral blood of healthy subjects were activated in vitro with anti-CD3 + CD28 mAbs in the presence or absence of 100 nM rapamycin. T cell proliferation was monitored by CFSE dilution at the end of one, two, and three rounds of stimulation (see Fig. 1 for a schematic description of the experiments). In the first 2 wk of culture, the ability to proliferate upon polyclonal activation was markedly reduced in T cells exposed to rapamycin compared with control cultures (average percentage of proliferating cells in the first week of culture: 1.9 ± 0.6 in rapamycin cultures; 23 ± 6.7 in medium cultures; $n = 6$) (Fig. 2*A*). By the third week of culture, T cells activated in the presence or absence of rapamycin proliferated to a similar extent. These data suggest that rapamycin-resistant T cells spared during the first two rounds of stimulation can subsequently expand in the presence of rapamycin and exogenous IL-2. Despite the T cell proliferation observed in the third week of culture with rapamycin, the number of T cells recovered in control cultures at the end of three rounds of stimulation (T-medium) was significantly higher (average fold expansion: 14 ± 5; $n = 14$) than in rapamycin cultures (T-rapamycin) (average fold expansion: 4 ± 2; $n = 14$) (Fig. 2*B*).

CD4$^+$ T cell proliferation was profoundly inhibited in the presence of rapamycin due to T cell arrest in G_1 phase (data not shown). Rapamycin, by binding to FKBP12, inhibits the function of mammalian target of rapamycin, which is involved in a broad range of physiological processes linked to the control of cell cycle (reviewed in Ref. 17). Similarly to what we observed in human

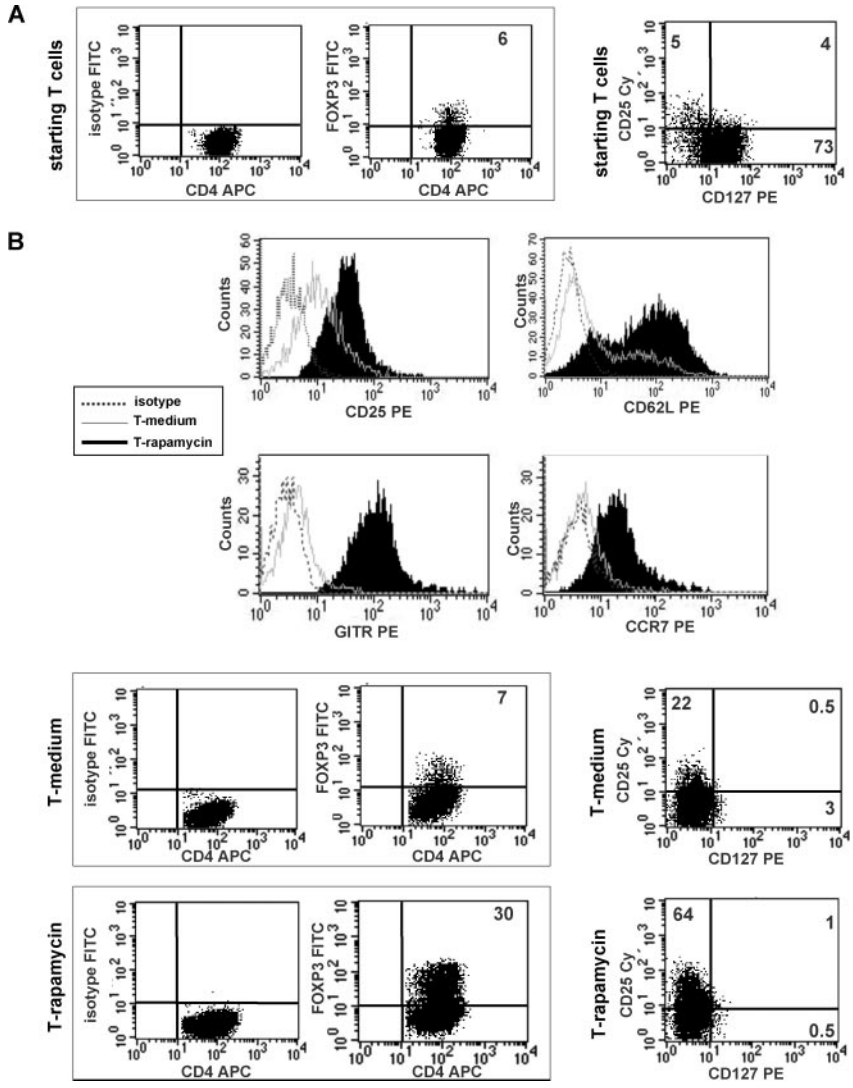

FIGURE 3. T-rapamycin $CD4^+$ T cells express regulatory markers. *A*, Expression of CD4/FOXP3 with the relative isotype control and of CD127/CD25 in freshly isolated $CD4^+$ T cells before culture was tested by FACS. One representative experiment of eight is presented. Quadrants were set in the dot plots based on the internal isotype controls. *B*, After three rounds of stimulation in the absence (T-medium) or presence of rapamycin (T-rapamycin), the cells were left resting for 1 wk in the presence of IL-2 (100 U/ml) and in the absence of rapamycin with no further stimulation. After 7 days, cells were analyzed by FACS. One representative experiment of eight is presented. Numbers are percentages of positive cells in that quadrant.

cells, we reported that $CD4^+$ T cells isolated from spleens of wild-type and TCR transgenic mice and activated in the presence of 100 nM rapamycin had a delayed kinetic of proliferation compared with control cells. However, at the end of the third week of culture, the same number of murine T cells was recovered in control and rapamycin cultures (18). This difference can be due to a different sensitivity of murine and human T cells to rapamycin.

We further investigated whether rapamycin promotes T cell death and/or T cell anergy in vitro. To this purpose, propidium iodide (PI), which crosses the plasma membrane of nonviable cells, was combined with CFSE staining. $CD4^+$ T cells that did not proliferate upon polyclonal activation were largely PI^+ in both medium (average undivided PI^+ cells: $70 \pm 3\%$; $n = 3$) and rapamycin culture (average undivided PI^+ cells: $84 \pm 5\%$; $n = 3$) (Fig. 2*C*). However, the overall number of undivided PI^+ T cells was higher in rapamycin cultures because of the marked decreased in cell proliferation. After three rounds of stimulation, $CD4^+$ T cells activated in the presence of rapamycin and restimulated in the absence of the compound and of exogenous IL-2 retained their ability to proliferate, indicating that rapamycin does not induce T cell anergy (Fig. 2*D*). However, the overall number of cell divisions was slightly reduced compared with control cells. Collectively, these data demonstrate that the majority of rapamycin-sensitive $CD4^+$ T cells die upon activation in the presence of 100 nM rapamycin, whereas those that are spared by rapamycin are not anergic and efficiently proliferate upon subsequent restimulation. These data are in agreement with those recently published by Nikolaeva et al. (19), who demonstrated that peripheral blood from healthy individuals cocultured with irradiated allogeneic cells in a MLC in the presence of rapamycin are not anergic once restimulated in the absence of the compound.

$CD4^+$ T cells expanded in the presence of rapamycin are $CD25^+FOXP3^+$ and are suppressive

Four to 10% of $CD4^+$ T cells freshly isolated from peripheral blood constitutively expressed FOXP3 (Fig. 3*A*). As recently demonstrated (10, 11), the majority of $CD4^+CD25^+$ T cells, especially the $CD25^{high}$ subset, was $CD127^{low/-}$ (Fig. 3*A*). Phenotype of these T cells activated in vitro for 3 wk in the presence or absence of rapamycin was analyzed after 1 wk of resting in IL-2 and in the absence of any compound. T-rapamycin cells constitutively expressed high levels of CD25, CD62L, GITR, CCR7, and FOXP3, whereas T-medium cells did not (Fig. 3*B*). There is increasing evidence that FOXP3 expression can be induced upon activation in human $CD4^+CD25^-$ T effector cells in both fresh T cells and T cell lines/clones (20). Accordingly, in our experimental system, FOXP3 expression was up-regulated at the end of three rounds of stimulation also in T-medium cells (data not shown). However, after 1 wk of resting, FOXP3 in T-medium cells returned to levels similar to those of freshly isolated $CD4^+$ T cells, whereas in

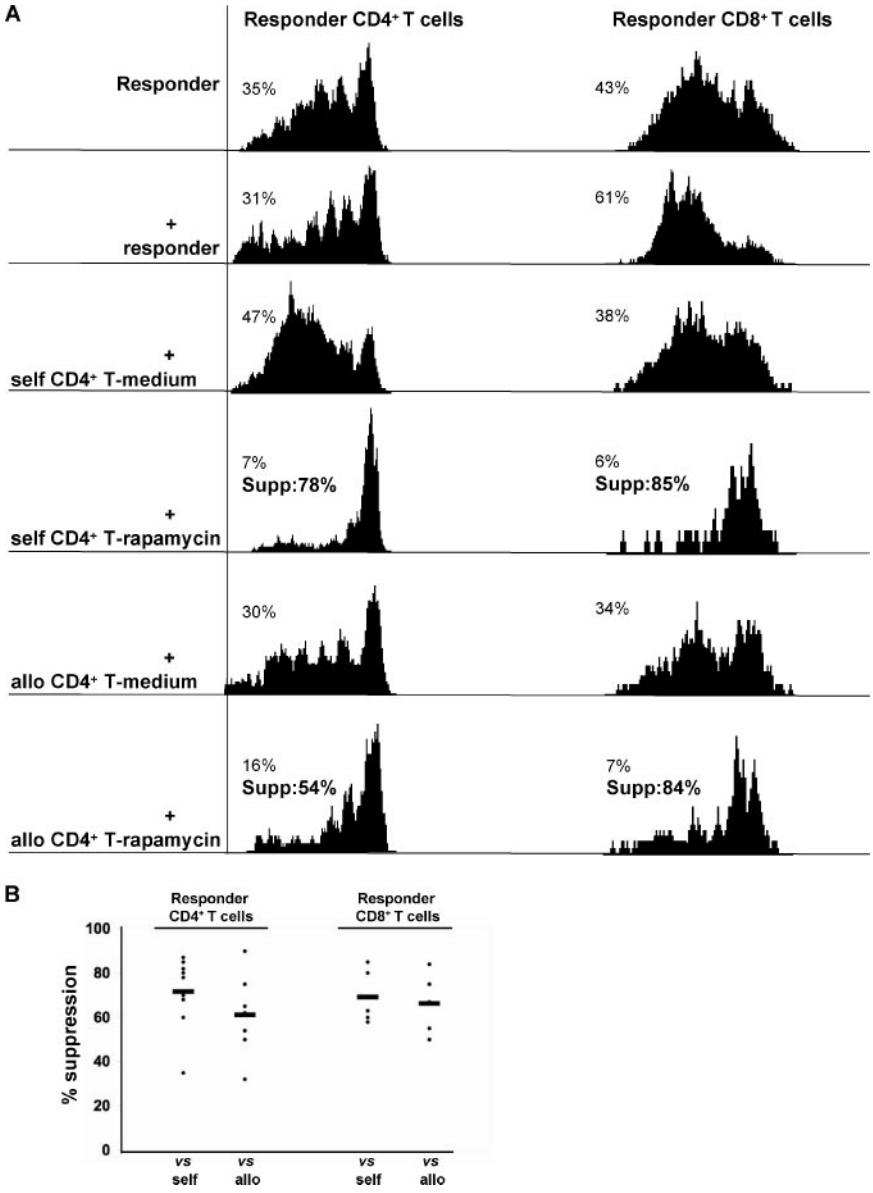

FIGURE 4. T-rapamycin CD4$^+$ T cells suppress proliferation of syngeneic and allogeneic CD4$^+$ and CD8$^+$ T cells. *A*, Purified CD4$^+$ T cells freshly isolated from healthy subjects were stained with CFSE and were activated with anti-CD3 + anti-CD28 mAbs (responder CD4$^+$ T cells; *left panel*). Alternatively, CD4$^-$ cells isolated from healthy subjects were stained with CFSE and were activated with anti-CD3 + anti-CD28 mAbs. Proliferation of CD8$^+$ T cells present in CD4$^-$ cells was tracked upon staining with anti-CD8 mAb at the moment of FACS analysis (responder CD8$^+$ T cells; *right panel*). CD4$^+$ T cells syngeneic (self) or allogeneic (allo) to responder cells and obtained after 3-wk culture in the absence (T-medium) or presence of rapamycin (T-rapamycin) were added in equal number to responder CFSE$^+$ T cells (10^5:10^5). After 7 days of culture, cell division was measured by CFSE dilution. Histograms show the FACS profile of CFSE$^+$ T cells. The amount of CFSE$^+$ T cells proliferating in the absence or presence of cultured T cells was calculated, as described in *Materials and Methods*, and percentages of divided cells in each culture condition are indicated. Percentages of suppression in comparison with proliferation of responder cells are indicated. One representative experiment is shown. *B*, Percentage of suppression mediated by T-rapamycin cells obtained in each experiment is presented. Each dot represents one experiment. Lines represent average of suppression. There were no statistically significant differences between suppression vs self and allo-T cells.

T-rapamycin cells FOXP3 remained constitutively high (Fig. 3*B*). Repetitive activation of T cells in the presence or absence of rapamycin led to down-regulation of CD127 (Fig. 3*B*).

It has been shown that alloantigen-driven expansion of human CD4$^+$CD25$^+$ Tregs gives rise to a distinct highly suppressive subset of CD27$^+$ Tregs next to a moderately suppressive CD27$^-$ Treg subset. Rapamycin, on the contrary to cyclosporin A, was shown to preserve the dominance of the potent CD27$^+$ Tregs over the CD27$^-$ cells (21). In our T-rapamycin cells, CD27 was never overexpressed compared with T-medium cells (data not shown). This discrepancy can be due to the different stimuli used in the two culture systems.

T-rapamycin cells suppressed T cell proliferation of syngeneic and allogeneic CD4$^+$ and CD8$^+$ T cells activated with anti-CD3 + anti-CD28 mAbs, whereas control T-medium cells did not inhibit T cell proliferation in any condition tested (Fig. 4*A*). The experiments were reproducible between different donors, and average of suppression of syngeneic CD4$^+$ T cell proliferation was 71 $\pm$ 16% (n = 9), and suppression of syngeneic CD8$^+$ T cell proliferation was 69 $\pm$ 12% (n = 5). There was no significant difference in the ability to suppress syngeneic vs allogeneic T cell proliferation (Fig. 4*B*). In our experimental system, T-rapamycin cells suppress CD4$^+$ T cell proliferation via a direct T-T cell mechanism, whereas suppression of CD8$^+$ T cell proliferation could also be ascribed to an indirect mechanism. CD8$^+$ T cells used in the suppression assays were indeed not purified cells, but were obtained as the negative fraction after purification of CD4$^+$ T cells (average CD8$^+$ T cell content: 42 $\pm$ 14.1%; n = 9). An indirect effect of T-rapamycin cells on APCs present in the CD4$^-$ cell preparations cannot therefore be excluded.

Rapamycin selectively blocks expansion of CD4$^+$CD25$^-$ T effector cells, whereas it allows CD4$^+$CD25$^+$ Treg growth

To define whether rapamycin depletes CD4$^+$CD25$^-$ T effector cells from the culture leading to a selective enrichment of CD4$^+$CD25$^+$ Tregs, we performed experiments in which we could specifically track either CD4$^+$CD25$^-$ T effector cells or CD4$^+$CD25$^+$ Tregs. To this purpose, peripheral CD4$^+$ T cells were FACS sorted in CD25$^-$ and CD25$^+$ T cell subsets (purity $\geq$95%, and FOXP3 expression was 0 and 98% in CD25$^-$ and CD25$^+$ T cells, respectively). To maintain the same culture conditions previously described, one of the

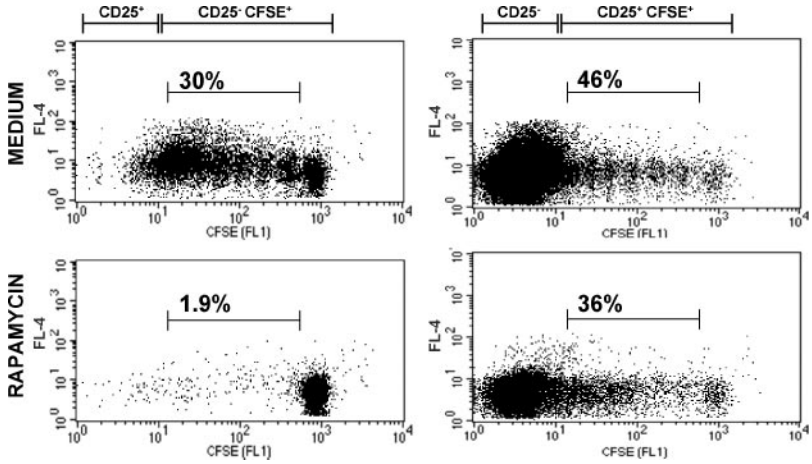

FIGURE 5. Rapamycin selectively blocks proliferation of $CD4^+CD25^-$ T cells, but not of $CD4^+CD25^+$ Tregs. $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells were FACS sorted. Subsequently, sorted $CD4^+CD25^-$ T cells were stained with CFSE and sorted unstained $CD4^+CD25^+$ T cells were added back in the same proportion present in total $CD4^+$ T cells (5%) (*left panels*). Alternatively, sorted $CD4^+CD25^+$ T cells were stained with CFSE and unstained sorted $CD4^+CD25^-$ T cells were added back in the same proportion present in total $CD4^+$ T cells (95%) (*right panels*). The two distinct CFSE-stained cell populations were activated with anti-CD3 + anti-CD28 mAbs + IL-2 (100 U/ml) in the presence or absence of 100 nM rapamycin. CFSE dilution was monitored 7 days after activation. Numbers indicate the percentage of $CFSE^+$ dividing cells. One representative experiment of two is presented.

two sorted population was CFSE stained and subsequently remixed to the unstained T cell counterpart in the original proportion (i.e., 95% $CD25^-$ cells and 5% $CD25^+$ cells). Cultures of $CD25^-CFSE^+$ plus $CD25^+CFSE^-$ cells and $CD25^-CFSE^-$ plus $CD25^+CFSE^+$ cells were activated with anti-CD3 + anti-CD28 mAbs and IL-2 in the presence or absence of rapamycin. Proliferation of CFSE-stained cells was tested 7 days after activation. T cell proliferation of $CD4^+CD25^-$ cells was strongly reduced by rapamycin, whereas $CD4^+CD25^+$ T cells were not significantly affected by the antiproliferative effect of the drug (Fig. 5). These data suggest that the presence of $CD4^+CD25^+$ Tregs with suppressive activity in rapamycin cultures is most likely due to a selective enrichment and expansion of the $CD4^+CD25^+$ Treg subset that is already present in limited amounts at the beginning of the culture and that is resistant to rapamycin.

Rapamycin promotes expansion of functional $CD4^+CD25^+FOXP3^+$ Tregs from T1D patients

Despite the original observation made by Kukreja et al. (22) that $CD4^+CD25^+$ T cells are reduced in T1D subjects, it is now ascertained that the frequency of $CD4^+CD25^{+/bright}$ T cells in T1D patients is similar to that of healthy controls (12, 13, 23). We observed that, similarly to $CD4^+CD25^+$ T cell frequency, FOXP3 expression was superimposable in $CD4^+CD25^{bright}$ T cells of T1D and ND (Fig. 6). Thus, T1D individuals not only have a frequency of $CD4^+CD25^{bright}$ T cells equivalent to healthy individuals, but this subset also expresses comparable levels of FOXP3 and CD127 (data not shown), confirming the data recently published by Liu et al. (10).

To define whether rapamycin is able to promote expansion of functional $CD4^+CD25^+FOXP3^+$ Tregs also from patients with ongoing autoimmune disease, $CD4^+$ T cells isolated from peripheral blood of T1D patients were activated in the presence or absence of rapamycin. The phenotype of T1D $CD4^+$ T cells cultured in the presence of rapamycin for 3 wk and then in medium with IL-2 for 1 wk was similar to that of ND. T1D T-rapamycin cells constitutively expressed high levels of CD25, CD62L, GITR, CCR7, and FOXP3, whereas control T-medium cells did not. Similarly to ND, the expression of CD127 was lost in both T-medium and T-rapamycin cells (Fig. 7). T1D T-rapamycin cells were then

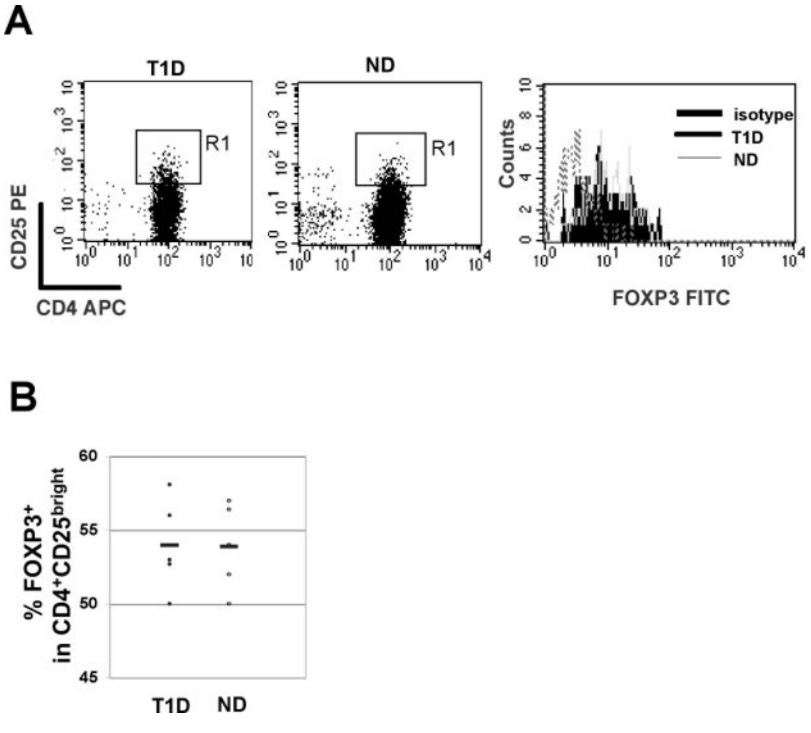

FIGURE 6. $CD4^+$ T cells of ND and T1D patients express similar levels of FOXP3. *A*, FOXP3 expression was tested by FACS in $CD4^+CD25^{bright}$-gated T cells (R1 gate shown in the dot plots) of T1D patients and ND. One representative FOXP3 histogram, on R1-gated cells, is shown. *B*, Percentage of $FOXP3^+$ cells within $CD4^+CD25^{bright}$ T cells in each T1D patients ($n = 5$) and ND tested ($n = 5$) is shown. Each dot represents one donor. Lines represent average of $CD4^+CD25^{bright}$ T cells expressing FOXP3. There were no statistically significant differences between FOXP3-expressing $CD4^+CD25^{bright}$ T cells in ND vs T1D subjects.

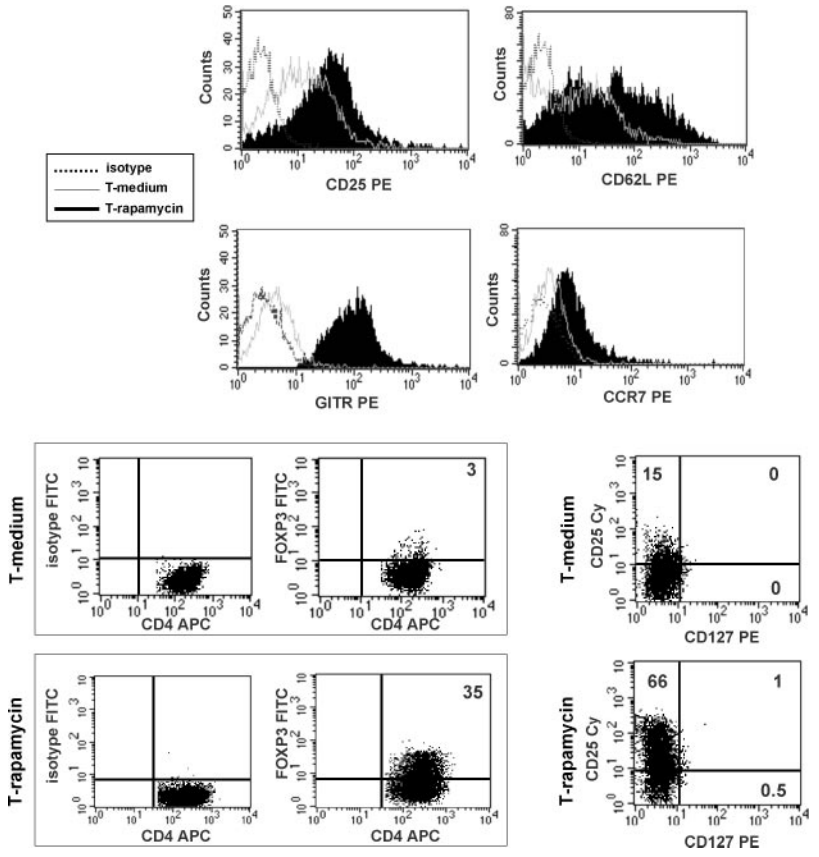

FIGURE 7. T-rapamycin CD4+ T cells from T1D patients express regulatory markers. After three rounds of stimulation in the absence (T-medium) or presence of rapamycin (T-rapamycin), the cells were left resting for 1 wk in the presence of IL-2 (100 U/ml) and in the absence of rapamycin with no further stimulation. After 7 days, cells were analyzed by FACS. One representative experiment of five is presented. Quadrants were set in the dot plots based on the internal isotype controls. Numbers are precentages of positive cells in that quadrant.

tested for their ability to suppress proliferation of allogeneic responder CD4+ T cells in vitro but not of syngeneic CD4+ T cells, which were not available, and of syngeneic and allogeneic responder CD8+ T cells. Their suppressive ability was compared with that of ND T-rapamycin cells, used as internal controls in the same experiments. CD4+ T cells from T1D patients expanded in the presence of rapamycin were as effective as ND T-rapamycin cells in suppressing proliferation of both CD4+ and CD8+ T cells (Fig. 8*A*). In one T1D patient, the T-rapamcyin cells did not suppress proliferation of syngeneic CD8+ T cells, whereas the ability to suppress allogeneic CD4+ and CD8+ T cell proliferation was preserved (Fig. 8*B*).

Overall, these data indicate that there is no reduced frequency of CD4+CD25+FOXP3+ Tregs in T1D subjects. More interestingly, cells from all patients tested suppress in vitro proliferation of allogeneic CD4+ T cells after expansion in the presence of rapamycin, and cells from four of five patients tested suppress proliferation of both syngeneic and allogeneic CD8+ T cells.

Discussion

In this study, we have shown that rapamycin markedly reduces human CD4+ T cell proliferation in vitro, but does not induce T cell anergy. Interestingly, CD4+CD25− T effector cells are the ones highly sensitive to the antiproliferative effect of rapamycin, whereas CD4+CD25+FOXP3+ Tregs are resistant and efficiently expand in the presence of the drug. Accordingly, CD4+CD25+FOXP3+ Tregs with suppressive ability expand upon activation in the presence of rapamycin. Important for potential clinical use, rapamycin promotes expansion of functional Tregs also from T1D patients.

Preclinical murine models have shown that rapamycin, on the contrary to calcineurin inhibitors, enhances Treg proliferation and regulatory function in vivo (24, 25). Recently, it has been demonstrated by two independent studies that rapamycin allows the formation of human regulatory CD4+CD25bright T cells in vitro in MLC (19, 26). However, in the above mentioned studies, CD4+CD25bright Tregs were also generated in MLC in the absence of rapamycin. Furthermore, these MLC Tregs needed to be sorted to display an in vitro suppressive activity. These data clearly indicate that adaptive Tregs are induced during MLC and that rapamycin does not prevent this induction. Unfortunately, in these studies, the cytokine production and the mechanisms of suppression of Tregs were not investigated. It is therefore unclear whether the adaptive Tregs described in these studies are similar to the in vitro induced Tregs (27) or Treg type 1 cells, which up-regulate CD25 and FOXP3 upon in vitro activation (28). In addition, the adaptive Tregs described by Nikolaeva et al. (19) and Valmori et al. (26) are distinct from the Tregs generated in our study, in which CD4+ T cells cultured in the absence of rapamycin never displayed a suppressive activity in any experiment performed. At the moment it cannot be excluded that rapamycin can both expand naturally occurring Tregs and induce adaptive Tregs from human CD4+ T cells.

The IL-2-mediated proliferation and survival of activated T cells require a coordinate effort between multiple signaling pathways downstream of the IL-2R. The initial events upon IL-2 binding result in activation of the transcription factor STAT5 as well as recruitment of PI3K and Ras-MAPK. This complex signaling system ultimately results in the up-regulation of genes critical for cell cycle progression and survival. It is now clear that the IL-2-IL-2R signaling pathway is fundamental for the development and peripheral activity of Tregs (reviewed in Ref. 29). It is therefore unexpected that rapamycin, which inhibits signal transduction delivered by IL-2, allows expansion of functional Tregs. It has been shown that engagement of the IL-2R on CD4+CD25+ Tregs fails to activate downstream targets of the PI3K signaling pathway, which is

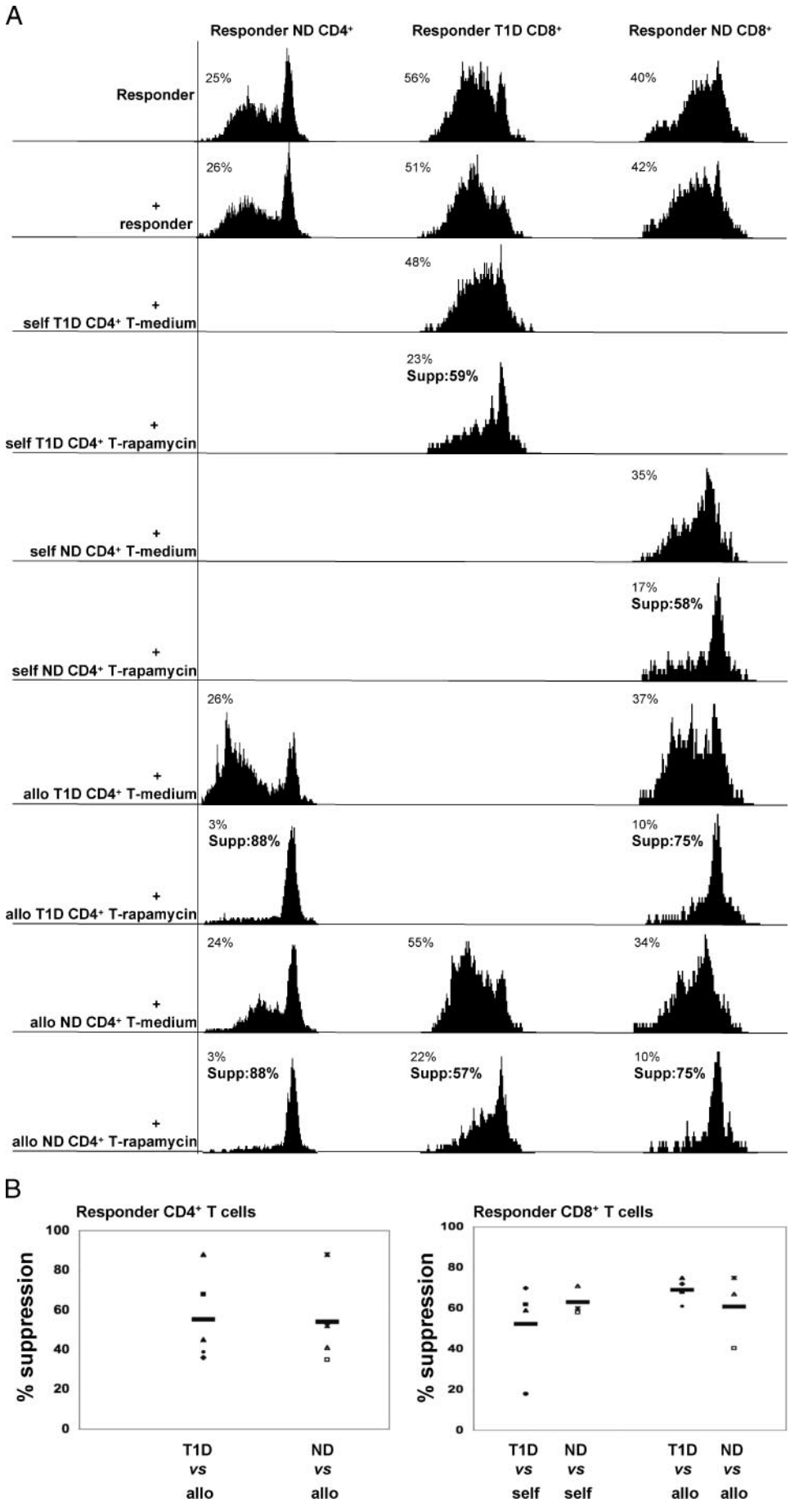

FIGURE 8. T-rapamycin $CD4^+$ T cells from T1D patients suppress proliferation of $CD4^+$ and $CD8^+$ T cells. *A*, $CD4^+$ T cells freshly isolated from ND were stained with CFSE and were activated with anti-CD3 + anti-CD28 mAbs (responder $CD4^+$ T cells; *left panel*). Alternatively, $CD4^-$ cells isolated from T1D patients (*middle panel*), or ND (*right panel*) were stained with CFSE and were activated with anti-CD3 + anti-CD28 mAbs. Proliferation of $CD8^+$ T cells present in $CD4^-$ cells was tracked upon staining with anti-CD8 mAb at the moment of FACS analysis (responder $CD8^+$ T cells; *middle and right panels*). $CD4^+$ T cells syngeneic (self) or allogeneic (allo) to responder cells obtained after 3 wk of culture in the absence (T-medium) or presence of rapamycin (T-rapamycin) were added in equal number to responder $CFSE^+$ T cells (10^5:10^5). After 7 days of culture, cell division was measured by CFSE dilution. Histograms show the FACS profile of $CFSE^+$ T cells. The amount of $CFSE^+$ cells proliferating in the absence or presence of cultured T cells was calculated, as described in *Materials and Methods*, and percentages of divided cells in each culture condition are indicated. Percentages of suppression in comparison with proliferation of responder cells are indicated. One representative experiment is shown. *B*, Percentage of suppression mediated by T-rapamycin cells obtained in each experiment is presented. Each dot represents one experiment. Lines represent average of suppression. There were no statistically significant differences between suppression of T1D T-rapamycin cells vs ND T-rapamycin cells.

instead functional in CD4$^+$CD25$^-$ T cells (30). Therefore, we hypothesize that rapamycin, by binding mammalian target of rapamycin and selectively blocking PI3K-mediated signaling, specifically targets PI3K-sensitive CD4$^+$CD25$^-$ T effector cells while sparing CD4$^+$CD25$^+$ Tregs. In support of this hypothesis, Zorn et al. (31) recently demonstrated that the IL-2-mediated up-regulation of FOXP3 in purified CD4$^+$CD25$^+$ T cells involves the binding of STAT3/5 proteins to a highly conserved STAT binding site located in the first intron of the FOXP3 gene. It is therefore possible that rapamycin, by sparing the JAK/STAT-IL-2 signaling pathway, consequently maintains/induces high FOXP3 expression.

T cells lacking prosurvival proteins Pim-1 and Pim-2 are highly sensitive to the antiproliferative effect of rapamycin (32). A possible selective Pim1/2 expression in CD4$^+$CD25$^+$ Tregs might also explain the different sensitivity to rapamycin. Furthermore, CD4$^+$CD25$^+$ Tregs may undergo less apoptosis than CD4$^+$CD25$^-$ T effector cells once activated in the presence of rapamycin (L. Strauss, T. L. Whiteside, A. Knights, C. Bergmann, A. Knut, and A. Zippelius, submitted for publication).

The existence of CD4$^+$CD25$^+$ Tregs with defective suppressor function in T1D patients has been demonstrated by Lindley et al. (13) and Brusko et al. (12), whereas Putnam et al. (23) showed that T1D CD4$^+$CD25$^+$ Tregs are as suppressive as those of ND. Among the factors that could account for the discrepancies in these studies is the different purity of the tested Tregs. The study by Putnam et al. (23) was performed using FACS-sorted CD4$^+$CD25^{bright} T cells, whereas Lindley et al. (13) and Brusko et al. (12) used beads-sorted CD4$^+$CD25$^+$ T cells. It is therefore possible that T1D patients do not have defective CD4$^+$CD25$^+$ Tregs per se; rather, their beads-sorted CD4$^+$CD25$^+$ T cells have a higher frequency of contaminating T effector cells compared with those of healthy individuals. The reported increased expression of activation markers (namely CD69 and HLA-DR) on CD4$^+$ and CD8$^+$ T cells in T1D patients, both at onset and during the course of the disease, is consistent with this hypothesis (33, 34). Thus, only highly pure FACS-sorted CD4$^+$CD25^{bright} T cells display suppressive function in vitro (23). Alternatively, in vitro culture of CD4$^+$ T cells in the presence of rapamycin might deplete contaminating T effector cells while sparing functional Tregs with suppressive ability comparable to that of ND.

The assumption that CD4$^+$CD25$^+$ Tregs freshly isolated from peripheral blood of T1D patients are equivalent to rapamycin-expanded CD4$^+$CD25$^+$FOXP3$^+$ Tregs cannot, at this moment, be made. We cannot exclude that a defect in freshly isolated T1D CD4$^+$CD25$^+$ Tregs is present and that in vitro culture can functionally modify these cells, as we observed in patients affected by immune dysregulation-polyendocrinopathy-entheropathy-X-linked disease (IPEX) (35). Alternatively, rapamycin might induce adaptive CD4$^+$CD25$^+$FOXP3$^+$ Tregs from peripheral human CD4$^+$ T cells, on the contrary to what we have shown previously in the mouse (18).

Due to the lack of cells, we could not determine whether T-rapamycin cells from T1D patients suppress proliferation of syngeneic CD4$^+$ T cells in vitro. The ability to suppress proliferation of syngeneic CD8$^+$ T cells by T1D T-rapamycin cells was preserved in all patients tested, except one. Of note is that CD8$^+$ responder T cells of this patient produced 2–3 times more IFN-γ upon activation compared with CD8$^+$ T cells isolated from the other patients tested (data not shown). This suggests that the stage of activation of CD8$^+$ T cells from T1D patients may determine their ability to be suppressed in vitro. We have indeed recently shown that in IPEX patients with FOXP3 mutation and T1D, the level of suppression depends not only on the function of Tregs, but also on the T effector cells (35).

It is important to highlight that the T cells used in this study were isolated from T1D patients who were diagnosed relatively recently (i.e., average disease duration: 16.5 mo). It remains to be determined whether T cells isolated from T1D patients with long-term disease have a similar response to rapamycin. Experiments are currently ongoing to address this question.

Rapamycin-expanded Tregs of both ND and T1D patients constitutively express FOXP3, GITR, CD62L, and CCR7. Expression of CD62L and CCR7 crucially determines the lymph node-homing capacity of cells, including CD4$^+$CD25$^+$ Tregs (36). Only CD4$^+$CD25$^+$CD62L$^+$CCR7$^+$ Tregs migrate to pancreatic lymph nodes and delay diabetes onset in an adoptive transfer setting (37). Thus, in vitro Treg expansion protocols that preserve the expression of CD62L and CCR7 may be more effective than those that do not. It has been demonstrated recently that the CD127 can be used as a biomarker for human Tregs. CD4$^+$CD25^{high}FOXP3$^+$ Tregs are indeed CD127$^{-/low}$ (10, 11). Human CD4$^+$ T cells from both ND and T1D activated in vitro for 3 wk with anti-CD3 + CD28 mAbs are all CD127$^-$ irrespective of the presence or absence of rapamycin. Therefore, after in vitro culture, CD127 cannot be used as a biomarker to discriminate between Tregs and T effector cells.

Recently, improved and clinically scalable culture conditions that should permit pilot trials of CD4$^+$CD25$^+$ Treg adoptive immunotherapy for the prevention or cure of graft vs host disease after bone marrow transplantation have been proposed (38, 39). These protocols require the following: 1) highly purified sorted CD4$^+$CD25^{bright} Tregs to avoid CD25$^+$ T effector cell contamination, and 2) high doses of exogenous IL-2 to break the anergic Treg phenotype. Our findings suggest that stringent sorting conditions are not necessary and Treg expansion in the presence of rapamycin might represent a novel and safe cellular immunotherapy protocol for the cure of T cell-mediated disease.

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Disclosures

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References

1. Kronenberg, M., and A. Rudensky. 2005. Regulation of immunity by self-reactive T cells. *Nature* 435: 598–604.
2. Sakaguchi, S. 2005. Naturally arising Foxp3-expressing CD25$^+$CD4$^+$ regulatory T cells in immunological tolerance to self and non-self. *Nat. Immunol.* 6: 345–352.
3. Levings, M. K., R. Sangregorio, and M. G. Roncarolo. 2001. Human CD25$^+$CD4$^+$ T regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *J. Exp. Med.* 193: 1295–1302.
4. Dieckmann, D., H. Plottner, S. Berchtold, T. Berger, and G. Schuler. 2001. Ex vivo isolation and characterization of CD4$^+$CD25$^+$ T cells with regulatory properties from human blood. *J. Exp. Med.* 193: 1303–1310.
5. Jonuleit, H., E. Schmitt, M. Stassen, A. Tuettenberg, J. Knop, and A. H. Enk. 2001. Identification and functional characterization of human CD4$^+$CD25$^+$ T cells with regulatory properties isolated from peripheral blood. *J. Exp. Med.* 193: 1285–1294.
6. Baecher-Allan, C., V. Viglietta, and D. A. Hafler. 2004. Human CD4$^+$CD25$^+$ regulatory T cells. *Semin. Immunol.* 16: 89–98.
7. Baecher-Allan, C., J. A. Brown, G. J. Freeman, and D. A. Hafler. 2001. CD4$^+$CD25^{high} regulatory cells in human peripheral blood. *J. Immunol.* 167: 1245–1253.
8. Levings, M. K., R. Sangregorio, C. Sartirana, A. L. Moschin, M. Battaglia, P. C. Orban, and M. G. Roncarolo. 2002. Human CD25$^+$CD4$^+$ T suppressor cell clones produce transforming growth factor β, but not interleukin 10, and are distinct from type 1 T regulatory cells. *J. Exp. Med.* 196: 1335–1346.
9. Ziegler, S. F. 2006. FOXP3: of mice and men. *Annu. Rev. Immunol.* 24: 209–226

10. Liu, W., A. L. Putnam, Z. Xu-Yu, G. L. Szot, M. R. Lee, S. Zhu, P. A. Gottlieb, P. Kapranov, T. R. Gingeras, B. F. de St Grothqq, et al. 2006. CD127 expression inversely correlates with FoxP3 and suppressive function of human $CD4^+$ T reg cells. *J. Exp. Med.* 203: 1701–1711.
11. Seddiki, N., B. Santner-Nanan, J. Martinson, J. Zaunders, S. Sasson, A. Landay, M. Solomon, W. Selby, S. I. Alexander, R. Nanan, et al. 2006. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J. Exp. Med.* 203: 1693–1700.
12. Brusko, T. M., C. H. Wasserfall, M. J. Clare-Salzler, D. A. Schatz, and M. A. Atkinson. 2005. Functional defects and the influence of age on the frequency of $CD4^+CD25^+$ T-cells in type 1 diabetes. *Diabetes* 54: 1407–1414.
13. Lindley, S., C. M. Dayan, A. Bishop, B. O. Roep, M. Peakman, and T. I. Tree. 2005. Defective suppressor function in $CD4^+CD25^+$ T-cells from patients with type 1 diabetes. *Diabetes* 54: 92–99.
14. Viglietta, V., C. Baecher-Allan, H. L. Weiner, and D. A. Hafler. 2004. Loss of functional suppression by $CD4^+CD25^+$ regulatory T cells in patients with multiple sclerosis. *J. Exp. Med.* 199: 971–979.
15. Kriegel, M. A., T. Lohmann, C. Gabler, N. Blank, J. R. Kalden, and H. M. Lorenz. 2004. Defective suppressor function of human $CD4^+CD25^+$ regulatory T cells in autoimmune polyglandular syndrome type II. *J. Exp. Med.* 199: 1285–1291.
16. Balandina, A., S. Lecart, P. Dartevelle, A. Saoudi, and S. Berrih-Aknin. 2005. Functional defect of regulatory $CD4^+CD25^+$ T cells in the thymus of patients with autoimmune myasthenia gravis. *Blood* 105: 735–741.
17. Abraham, R. T., and G. J. Wiederrecht. 1996. Immunopharmacology of rapamycin. *Annu. Rev. Immunol.* 14: 483–510.
18. Battaglia, M., A. Stabilini, and M. G. Roncarolo. 2005. Rapamycin selectively expands $CD4^+CD25^+FoxP3^+$ regulatory T cells. *Blood* 105: 4743–4748.
19. Nikolaeva, N., F. J. Bemelman, S. L. Yong, R. A. van Lier, and I. J. ten Berge. 2006. Rapamycin does not induce anergy but inhibits expansion and differentiation of alloreactive human T cells. *Transplantation* 81: 445–454.
20. Allan, S. E., L. Passerini, R. Bacchetta, N. Crellin, M. Dai, P. C. Orban, S. F. Ziegler, M. G. Roncarolo, and M. K. Levings. 2005. The role of 2 FOXP3 isoforms in the generation of human $CD4^+$ Tregs. *J. Clin. Invest.* 115: 3276–3284.
21. Coenen, J. J., H. J. Koenen, E. van Rijssen, L. B. Hilbrands, and I. Joosten. 2006. Rapamycin, and not cyclosporin A, preserves the highly suppressive $CD27^+$ subset of human $CD4^+CD25^+$ regulatory T cells. *Blood* 107: 1018–1023.
22. Kukreja, A., G. Cost, J. Marker, C. Zhang, Z. Sun, K. Lin-Su, S. Ten, M. Sanz, M. Exley, B. Wilson, et al. 2002. Multiple immuno-regulatory defects in type-1 diabetes. *J. Clin. Invest.* 109: 131–140.
23. Putnam, A. L., F. Vendrame, F. Dotta, and P. A. Gottlieb. 2005. $CD4^+CD25^{high}$ regulatory T cells in human autoimmune diabetes. *J. Autoimmun.* 24: 55–62.
24. Battaglia, M., A. Stabilini, E. Draghici, B. Migliavacca, S. Gregori, E. Bonifacio, and M. G. Roncarolo. 2006. Induction of tolerance in type 1 diabetes via both $CD4^+CD25^+$ T regulatory cells and T regulatory type 1 cells. *Diabetes* 55: 1571–1580.
25. Zeiser, R., V. H. Nguyen, A. Beilhack, M. Buess, S. Schulz, J. Baker, C. H. Contag, and R. S. Negrin. 2006. Inhibition of $CD4^+CD25^+$ regulatory T-cell function by calcineurin-dependent interleukin-2 production. *Blood* 108: 390–399.
26. Valmori, D., V. Tosello, N. E. Souleimanian, E. Godefroy, L. Scotto, Y. Wang, and M. Ayyoub. 2006. Rapamycin-mediated enrichment of T cells with regulatory activity in stimulated $CD4^+$ T cell cultures is not due to the selective expansion of naturally occurring regulatory T cells but to the induction of regulatory functions in conventional $CD4^+$ T cells. *J. Immunol.* 177: 944–949.
27. Walker, M. R., B. D. Carson, G. T. Nepom, S. F. Ziegler, and J. H. Buckner. 2005. De novo generation of antigen-specific $CD4^+CD25^+$ regulatory T cells from human $CD4^+CD25^-$ cells. *Proc. Natl. Acad. Sci. USA* 102: 4103–4108.
28. Roncarolo, M.-G., S. Gregori, M. Battaglia, R. Bacchetta, K. Fleischhauer, and M. K. Levings. 2006. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol. Rev.* 212: 28–50.
29. Malek, T. R., and A. L. Bayer. 2004. Tolerance, not immunity, crucially depends on IL-2. *Nat. Rev. Immunol.* 4: 665–674.
30. Bensinger, S. J., P. T. Walsh, J. Zhang, M. Carroll, R. Parsons, J. C. Rathmell, C. B. Thompson, M. A. Burchill, M. A. Farrar, and L. A. Turka. 2004. Distinct IL-2 receptor signaling pattern in $CD4^+CD25^+$ regulatory T cells. *J. Immunol.* 172: 5287–5296.
31. Zorn, E., E. A. Nelson, M. Mohseni, F. Porcheray, H. Kim, D. Litsa, R. Bellucci, E. Raderschall, C. Canning, R. J. Soiffer, et al. 2006. IL-2 regulates FOXP3 expression in human $CD4^+CD25^+$ regulatory T cells through a STAT dependent mechanism and induces the expansion of these cells in vivo. *Blood* 108: 1571–1579.
32. Fox, C. J., P. S. Hammerman, and C. B. Thompson. 2005. The Pim kinases control rapamycin-resistant T cell survival and activation. *J. Exp. Med.* 201: 259–266.
33. Roncarolo, M. G., M. Zoppo, R. Bacchetta, C. Gabiano, C. Sacchetti, F. Cerutti, and P. A. Tovo. 1988. Interleukin-2 production and interleukin-2 receptor expression in children with newly diagnosed diabetes. *Clin. Immunol. Immunopathol.* 49: 53–62.
34. Gessl, A., and W. Waldhausl. 1998. Increased CD69 and human leukocyte antigen-DR expression on T lymphocytes in insulin-dependent diabetes mellitus of long standing. *J. Clin. Endocrinol. Metab.* 83: 2204–2209.
35. Bacchetta, R., L. Passerini, E. Gambineri, M. Dai, S. E. Allan, L. Perroni, F. Dagna-Bricarelli, C. Sartirana, S. Matthes-Martin, A. Lawitschka, et al. 2006. Defective regulatory and effector T cell functions in patients with FOXP3 mutations. *J. Clin. Invest.* 116: 1713–1722.
36. Forster, R., A. Schubel, D. Breitfeld, E. Kremmer, I. Renner-Muller, E. Wolf, and M. Lipp. 1999. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* 99: 23–33.
37. Szanya, V., J. Ermann, C. Taylor, C. Holness, and C. G. Fathman. 2002. The subpopulation of $CD4^+CD25^+$ splenocytes that delays adoptive transfer of diabetes expresses L-selectin and high levels of CCR7. *J. Immunol.* 169: 2461–2465.
38. Godfrey, W. R., Y. G. Ge, D. J. Spoden, B. L. Levine, C. H. June, B. R. Blazar, and S. B. Porter. 2004. In vitro-expanded human $CD4^+CD25^+$ T-regulatory cells can markedly inhibit allogeneic dendritic cell-stimulated MLR cultures. *Blood* 104: 453–461.
39. Hoffmann, P., R. Eder, L. A. Kunz-Schughart, R. Andreesen, and M. Edinger. 2004. Large-scale in vitro expansion of polyclonal human $CD4^+CD25^{high}$ regulatory T cells. *Blood* 104: 895–8903.