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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[1]

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Rapamycin is an immunosuppressive drug currently used in different clinical settings. Although the capacity of rapamycin to inhibit the mammalian target of rapamycin serine/threonine protein kinase and therefore T cell cycle progression is well known, its effects are complex and not completely understood. It has been reported recently that TCR-mediated stimulation of murine CD4$^+$ T cells in the presence of rapamycin results in increased proportions of CD4$^+$ T cells with suppressive functions, suggesting that the drug may also exert its immunosuppressive activity by promoting the selective expansion of naturally occurring CD4$^+$ regulatory T cells (Treg). In this study, we show that stimulation of human circulating CD4$^+$ T cells in the presence of rapamycin results indeed in highly increased suppressor activity. By assessing the effect of rapamycin on the growth of nonregulatory and Treg populations of defined differentiation stages purified ex vivo from circulating CD4$^+$ T cells, we could demonstrate that this phenomenon is not due to a selective expansion of naturally occurring Tregs, but to the capacity of rapamycin to induce, upon TCR-mediated stimulation, suppressor functions in conventional CD4$^+$ T cells. This condition, however, is temporary and reversible as it is dependent upon the continuous presence of rapamycin. *The Journal of Immunology,* **2006, 177: 944–949.**

Rapamycin is an antibiotic with potent immunosuppressive properties (1) discovered ~30 years ago as the active principle produced by a strain of *Streptomyces hygroscopicus* (2). In mammalian cells, rapamycin binds to FK506-binding protein-12, a highly conserved cytoplasmic receptor. The FK506-binding protein-12-rapamycin complex then binds to and inhibits the kinase activities of the mammalian target of rapamycin serine/threonine protein kinase, the activation of which is essential for protein synthesis and cell cycle progression. The presence of rapamycin during T cell activation blocks T cell cycle progression in the G_1 phase, resulting in the induction of tolerance (3, 4). Because of its immunosuppressive properties, rapamycin is currently used to prevent graft rejection (5). In contrast, the cytostatic properties of rapamycin have also promoted its use, as well as that of other mammalian target of rapamycin inhibitors, for cancer therapy (6–8). As illustrated by these diverse clinical applications, the full spectrum of the effects of in vivo treatment with rapamycin and related compounds remains to date to be fully elucidated. Among unpredicted effects of rapamycin, Tian et al. (9) have reported a reduction of CD4$^+$CD8$^+$ double-positive thymocytes and increased proportions of CD4$^+$CD25$^+$ T cells in the periphery of rats treated with therapeutic doses of rapamycin. More recently, Battaglia et al. (10) have reported that stimulation of CD4$^+$ T cells from spleens of OVA TCR transgenic mice in the presence of rapamycin results in the selective expansion of naturally occurring CD4$^+$CD25$^+$ regulatory T cells (Tregs)[3] able to suppress allograft rejection in a model of allogeneic pancreatic islet transplantation. Based on these data, the authors have proposed that rapamycin may be used to selectively expand ex vivo naturally occurring Tregs for cellular therapy in T cell-mediated diseases. The relevance of these findings to the human system, however, has not been assessed to date. In addition, direct evidence of the ability of rapamycin to promote the selective expansion of naturally occurring human Tregs was not provided in previous studies.

CD4$^+$ Tregs play a key role in inducing and maintaining immunologic tolerance and are also involved in the modulation of immunoresponses to cancer, pathogens, and alloantigens (11–13). In humans, different subsets of CD4$^+$ Tregs have been described. They include Th3 cells secreting high levels of TGF-β1, which are induced by stimulation of CD4$^+$ T cells in the presence of TGF-β1 or by oral Ag administration and Tr-1 cells, secreting IFN-γ and IL-10, that can be induced following stimulation in the presence of IL-10 (14–16). The Th3 and Tr-1 subsets are generated in the periphery and allow the development of peripheral tolerance to self Ags not or poorly expressed in the thymus. A distinct subset is composed by naturally occurring Tregs, generated as a distinct

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[3] Abbreviations used in this paper: Treg, regulatory T cell; Nn, natural naive.

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lineage in the thymus and that constitutively express CD25. Thymically derived Tregs are constitutively anergic (17). The key role of cells from this subset in the maintenance of tolerance is demonstrated by the development of autoimmune diseases following their depletion or functional alteration in normal animals (18). The phenotypic characterization of naturally occurring Tregs among human circulating lymphocytes (the most readily available source of cells for immunological assessments in humans) has been complicated by the fact that CD25 is not exclusively expressed by Tregs, but also by nonregulatory lymphocytes, following activation. Furthermore, whereas initial studies in humans have focused on the overall $CD4^+CD25^+$ population that represents, as in mice, 6–10% of total $CD4^+$ T cells, later only $CD4^+$ T cells expressing the highest levels of CD25 ($CD4^+CD25^{bright}$) and with an Ag-experienced phenotype have been considered to be genuine Tregs. In addition to the previously described Ag-experienced Tregs (19) that most likely contain some proportions of peripherally derived Tregs and also of activated non-Tregs, we have recently identified a subset of clearly distinguishable $CD25^+$ cells contained in the naive $CD4^+CD45RA^+CCR7^+$ T cell fraction, which we have named natural naive (Nn) Tregs (20). NnTregs represent the naive circulating compartment of human naturally occurring Tregs. NnTregs display, in the presence of IL-2, a higher proliferative potential than Ag-experienced Tregs, but, similar to those, they are anergic and suppressor ex vivo. The definition of the circulating NnTreg subset highly facilitates the analysis of naturally occurring Tregs in humans.

In this study, we have assessed the effect of rapamycin on the growth, phenotype, and function of human circulating $CD4^+$ T cells, following TCR-mediated stimulation in the presence of IL-2. Consistent with the recent data obtained in mice (10), we have observed that stimulation of human $CD4^+$ T cells in the presence of rapamycin results in a highly increased suppressor function as compared with that of $CD4^+$ T cells stimulated in the absence of the drug. However, when we analyzed the origin of this phenomenon by assessing the effect of rapamycin on the growth and differentiation of non-Treg and Treg populations of defined differentiation stages, highly purified ex vivo from circulating $CD4^+$ T cells, we found that the increased suppressive activity in rapamycin-treated cultures is not due to the selective expansion of naturally occurring Tregs, but to rapamycin-induced anergy and suppressor functions in conventional $CD4^+$ T cell populations. This effect is dependent on the continuous presence of rapamycin in the cultures and is reversible upon withdrawal of the drug.

Materials and Methods

Samples, isolation of $CD4^+$ T cells, and cell sorting

Peripheral blood samples were obtained from the New York Blood Center. Mononuclear cells were isolated by density gradient sedimentation using a Ficoll-Hypaque gradient (Amersham Biosciences). $CD4^+$ T cells were enriched by magnetic cell sorting using the MiniMACS Separator (Miltenyi Biotec). For the cell sorting experiments, $CD4^+$ T cells were stained with anti-CD4 (BD Biosciences), anti-CD8, anti-CD45RA (Caltag Laboratories), and anti-CD25 (Beckman Coulter). After gating on the $CD4^+CD8^-$ lymphocytes, cells were separated into four subsets on the basis of CD45RA and CD25 expression, as described previously (20), using a FACSAria (BD Biosciences). Data analysis was performed using the FACSDiva software (BD Biosciences). The purity of sorted populations was routinely >96%.

Assessment of cell growth, differentiation, and suppression

To assess the growth potential of $CD4^+$ Treg and non-Treg populations in the absence or in the presence of rapamycin, total $CD4^+$ T cells or sorted T cell subsets were labeled or not with CFSE (5 μM; Molecular Probes) and stimulated with plate-bound anti-CD3 (OKT3; 0.4 μg/ml) and anti-CD28 (CD28.2; 1 μg/ml) (eBiosciences) in the presence of IL-2 (100 IU/ml), which was maintained throughout the culture period, and irradiated allogeneic $CD4^-CD8^-$ APCs. Where indicated, the culture was carried on in the presence of rapamycin (Sigma-Aldrich; 100 nM). Stimulation was repeated at weekly intervals. The phenotype of stimulated $CD4^+$ T cells was assessed at day 7 after stimulation by staining with Abs to CD3 (Caltag Laboratories), CD4 (BD Biosciences), CD45RA (Caltag Laboratories), CCR7 (BD Biosciences), and CD25 (Beckman Coulter). Cell division was assessed at day 5 after stimulation by FACS analysis of CFSE dilution. The mean cycle number was calculated as the sum of nf_n for $n = 0$ to $n$max, where n is the cycle number and f_n is the fraction of cells that have undergone n divisions. Cell growth was assessed at day 7 after stimulation by manual counting. The ability of Tregs to suppress the growth of responder total $CD4^+$ cells was assessed by coculture of CFSE-labeled responders (2×10^4 cells/well) with suppressors at a suppressor to responder cell ratio of 1:1, in 96-well U-bottom plates in the presence of 2×10^4 cells/well irradiated allogeneic $CD14^+$ cells as APCs and PHA (1 μg/ml; Sigma-Aldrich) or plate-bound anti-CD3/CD28 in the absence of exogenous IL-2. The growth (100 − percentage of undivided cells) in the wells with suppressor cells (experimental group) was compared with that in the wells without suppressors (control group). The percentage of growth was determined at day 5 after stimulation as follows: (growth of experimental group/growth of control) × 100.

Assessment of FOXP3 expression

RNA was prepared from in vitro-stimulated sorted $CD4^+$ T cell subsets on day 7 after stimulation using RNeasy Mini Kit (Qiagen). cDNA synthesis was performed using Promega Reverse Transcription System A3500 (Promega), and cDNA integrity was tested by amplification of RPL32 in a 35-cycle PCR. Semiquantitative FOXP3 mRNA expression was assessed using the following primers: forward primer, 5′-TCA CCT ACG CCA CGG TCA T-3′ and reverse primer, 5′-CAC AAA GCA CTT GTG CAG-3′. Quantitative real-time PCR was performed with a TaqMan assay on an ABI 7000 system (Applied Biosystems) using Assays-on-Demand Gene Expression probes for FOXP3 (Hs 00203958; Applied Biosystems). As a control of input cDNA, we used GAPDH, Taqman probe, and primers. The probe sequence was as follows: FAM-5′-AAG GTG AAG GTC GGA GTC AAC GGA TTT G-3′-TAMRA. Primer sequences for GAPDH were as follows: 5′-CCACATCGCTCAGACACCAT-3′ and 5′-CCAGGCGC CCAATACG-3′ (Applied Biosystems).

Results

Stimulation of human $CD4^+$ T cells in the presence of rapamycin results in increased proportions of $CD25^{bright}$ T cells and in increased suppressive activity

We initially assessed the effect of rapamycin on the growth and differentiation of total human circulating $CD4^+$ T cells. To this purpose, $CD4^+$ T cells were highly enriched from circulating lymphocytes of healthy donors using magnetic cell sorting and stimulated with plate-bound anti-CD3/CD28 Abs, APCs, and IL-2 in the absence or in the presence of rapamycin. To follow cell division, one part of the cells was labeled with CFSE before stimulation. Subsequently, the cultures underwent additional stimulation cycles, at 1-wk intervals. Cell division was measured at day 5 after stimulation, and total cell growth and phenotype were assessed at day 7 after stimulation. Cell division was clearly higher in the absence than in the presence of rapamycin, as reflected by both the percentage of undivided cells in the cultures and the mean cycle number (Fig. 1, *A–C*). At the end of each stimulation cycle, the total number of $CD4^+$ T cells recovered in control cultures was in average ~3- to 6-fold higher as compared with cultures in rapamycin (Fig. 1*D*). As assessed by staining with annexin V, 24 h after each stimulation, the presence of rapamycin had no detectable effect on activation-induced cell death (data not shown). At the end of the first stimulation cycle, $CD4^+$ T cells stimulated in the absence of rapamycin contained lower proportions of naive ($CD45RA^+CCR7^+$) and central memory $CD4^+$ T cells ($CD45RA^-CCR7^+$) as compared with those stimulated in the presence of rapamycin (Fig. 2, *A* and *B*). In contrast, the proportion of effector memory $CD4^+$ T cells ($CD45RA^-CCR7^-$) was higher in the absence than in the presence of rapamycin. After additional stimulation, both in the absence and in the presence of rapamycin,

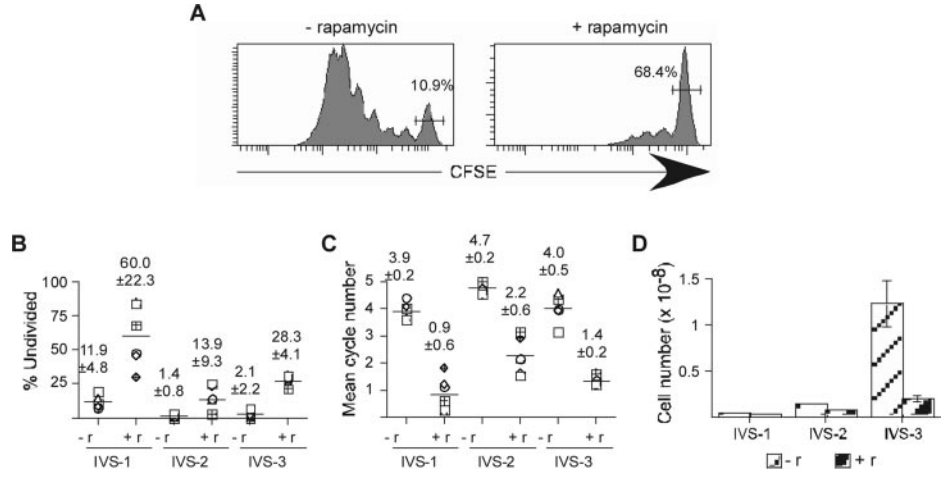

FIGURE 1. Rapamycin inhibits the proliferation of stimulated human CD4$^+$ T cells. CFSE-labeled or unlabeled CD4$^+$ T cells from healthy donors were stimulated with plate-bound anti-CD3/CD28 Abs, in the presence of allogeneic CD4$^-$CD8$^-$ APCs and in the presence or in the absence of rapamycin, for three weekly stimulation cycles. *A*, Example of cell growth of CD4$^+$ T cells in the absence or in the presence of rapamycin, at day 5 after the first stimulation. Cell division was measured based on CFSE dilution. The percentage of undivided cells in the cultures is shown. *B*, The percentage of undivided CD4$^+$ T cells at day 5 after each in vitro stimulation (IVS) was assessed on CFSE-labeled cultures. Data are shown for six donors as mean values including SD. Symbols identify individual donors. *C*, The mean number of cell divisions in each culture was assessed 5 days after each stimulation cycle on CFSE-labeled cultures and calculated as detailed in *Materials and Methods*. *D*, Cell growth was assessed by manual counting at day 7 after each stimulation cycle. Data are shown for six donors as mean values including SD.

however, the majority of the cells in the cultures acquired an effector memory phenotype (data not shown). Together these data point toward an effect of rapamycin in delaying T cell differentiation. CD4$^+$ T cells stimulated and cultured in the presence of rapamycin contained higher proportions of CD25^{bright} T cells as compared with control cultures. The increase of CD25^{bright} T cells in cultures containing rapamycin was already evident after the first stimulation cycle (Fig. 2, *C* and *D*) and was maintained after further stimulation. The presence of rapamycin in the absence of TCR-mediated stimulation had no effect on the cell cultures (data not shown). The suppressor capacity of CD4$^+$ T cells stimulated in vitro in the absence or in the presence of rapamycin was assessed 1 wk after the second cycle of stimulation. To this purpose, CD4$^+$ T cells from rapamycin-treated and control cultures were cocultured with allogeneic CFSE-labeled responder CD4$^+$ T cells and stimulated with either PHA or anti-CD3/CD28 Abs. As illustrated in Fig. 3, in both experimental settings, rapamycin-treated CD4$^+$ T cells displayed a capacity to suppress the proliferation of responder T cells that was much increased as compared with that of control cultures.

The increased suppressor function of rapamycin-treated cultures is not due to the selective expansion of naturally occurring Tregs

Battaglia et al. (10) have recently reported that the presence of CD4$^+$ T cells with increased suppressive activity in rapamycin-treated murine cultures is due to a selective expansion of the low proportions of naturally occurring CD4$^+$ Tregs present in the total CD4$^+$ T cell populations at the beginning of the cultures. To assess the relevance of these findings in our experimental system, we stained highly enriched CD4$^+$ T cells from healthy donors with Abs to CD45RA and CD25 and sorted them into four distinct subsets, as previously described (20): naive CD45RA$^+$CD25$^-$, NnTregs CD45RA$^+$CD25$^+$, and two Ag-experienced subsets, one CD45RA$^-$ expressing intermediate levels of CD25 (defined as

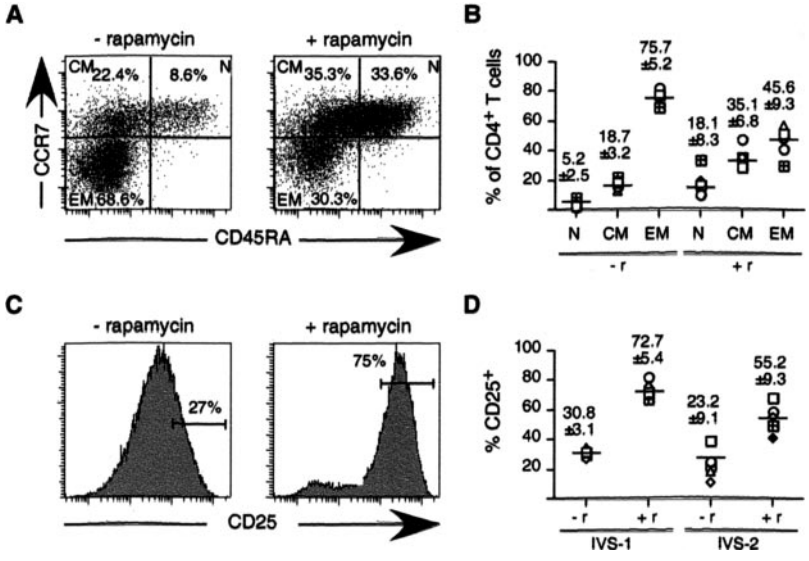

FIGURE 2. Rapamycin delays the differentiation of stimulated CD4$^+$ T cells and promotes the increase of CD4$^+$CD25^{bright} T cells in the cultures. The phenotype of CD4$^+$ T cells stimulated in the absence or in the presence of rapamycin was assessed at day 7 after the first cycle of stimulation by staining with the indicated Abs. *A*, Dot plots from one donor are shown on gated CD4$^+$ T cells. Numbers are percentage of cells in the corresponding quadrant. According to their expression of CD45RA and CCR7, populations were distinguished as naive (N, CD45RA$^+$CCR7$^+$), central memory (CM, CD45RA$^-$CCR7$^+$), and effector memory (EM, CD45RA$^-$CCR7$^-$). *B*, Data are shown for six donors as mean values including SD. Symbols identify individual donors. *C*, The expression level of CD25 was assessed on CD4$^+$ T cells stimulated in the absence or in the presence of rapamycin by staining with specific Abs. The percentage of CD25^{bright} T cells was determined as shown for one donor, on gated CD4$^+$ T cells. *D*, Data are presented for six donors.

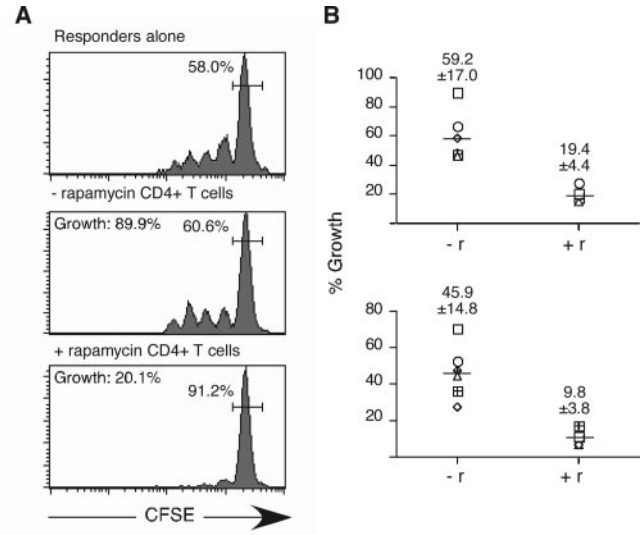

FIGURE 3. $CD4^+$ T cells stimulated and cultured in the presence of rapamycin exert suppressive activity. $CD4^+$ T cells from healthy donors cultured in the presence or in the absence of rapamycin during two stimulation cycles were assessed for their ability to suppress the proliferation of responder $CD4^+$ T cells following stimulation with PHA or anti-CD3/CD28 Abs. *A*, Suppressor and responder cell populations were mixed at a ratio of 1:1. Histograms show the FACS profiles of responder CFSE-labeled $CD4^+$ T cells. The percentage of undivided cells and percentage of cell growth with respect to the proliferation of control responder cells alone are indicated. Percentage of growth was calculated as detailed in *Materials and Methods*. *B*, Data obtained after stimulation with PHA (*upper panel*) or with anti-CD3/CD28 (*lower panel*) Abs are presented for six donors as mean values including SD.

$CD45RA^-CD25^-$), and the $CD45RA^-CD25^{bright}$ population corresponding to Ag-experienced Tregs (Fig. 4*A*). We have shown previously that both ex vivo suppressor functions and expression of FOXP3, a transcriptional repressor considered as a Treg marker, are confined to the Treg and NnTreg subsets (20). The sorted populations were stimulated with anti-CD3/CD28 Abs, IL-2, and APCs in the presence or absence of rapamycin, as detailed above. As illustrated in Fig. 4*B*, after the first cycle of stimulation, all sorted populations, with the exception of $CD45RA^-CD25^{bright}$ Tregs, which have poor proliferative capacity, had proliferated to a roughly similar extent in the absence of rapamycin. After additional stimulation in the absence of rapamycin, Ag-experienced $CD4^+CD25^-$ T cell populations proliferated slightly more than naive $CD4^+CD25^-$ T cells. Treg populations proliferated less than nonregulatory ones, although NnTregs proliferated more than Tregs (Fig. 4*B*). Proliferation of all populations was inhibited by the presence of rapamycin to an extent that was comparable or superior in regulatory as compared with nonregulatory populations (Fig. 4*B*). It is noteworthy that at the end of the first cycle of in vitro stimulation, both in the presence or in the absence of rapamycin, FOXP3 was expressed at high and comparable levels in all populations (Fig. 4*C*). These results are in line with recent data reported by Walker et al. (21), indicating that, at variance with the murine system, expression of FOXP3 in human conventional $CD4^+CD25^-$ T cells is induced following in vitro stimulation. Following in vitro expansion in the absence of rapamycin, the Ag-experienced $CD25^-$ populations were not suppressive, but instead they slightly facilitated the growth of cocultured $CD4^+$ T cells (Fig. 4*D*). We failed to detect significant suppressive activity with the populations derived from Ag-experienced Tregs. Interestingly, similar data (loss of suppressor function by Tregs following in vitro expansion) have been reported by Battaglia et al. (10), and are in line with the concept that Ag-experienced Treg populations contain variable proportions of non-Tregs that may overgrow after in vitro

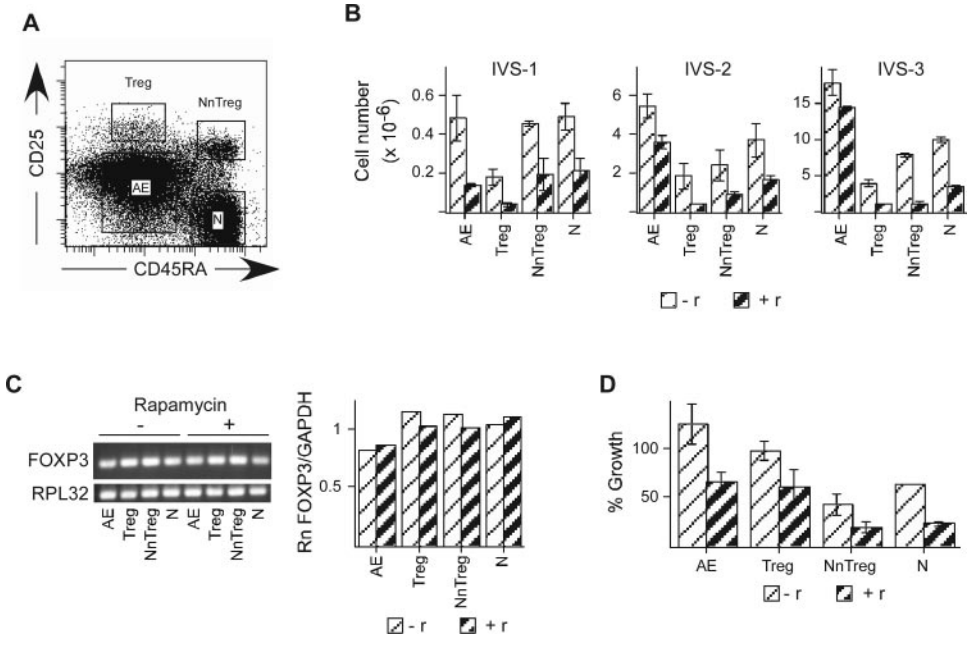

FIGURE 4. The suppressive activity in rapamycin-treated $CD4^+$ T cell cultures is not due to the selective expansion of naturally occurring Tregs. *A*, PBLs were stained with Abs to CD3, CD4, CD8, CD25, and CD45RA, and gated $CD4^+CD8^-$ T cells were sorted into four populations: conventional naive (N, $CD45RA^+CD25^-$), NnTregs ($CD45RA^+CD25^+$), conventional Ag-experienced (AE, $CD45RA^-CD25^{low}$), and Tregs ($CD45RA^-CD25^{bright}$), and stimulated with anti-CD3/CD28 Abs and IL-2 at weekly intervals. *B*, Cell growth was assessed by manual counting at day 7 after each stimulation cycle. Data are shown for two donors as mean values including SD. *C*, *FOXP3* gene expression was assessed by conventional and by quantitative real-time PCR on the four sorted populations after 1 wk of stimulation in the absence or in the presence of rapamycin. *D*, Suppression of $CD4^+$ T cell growth by the four sorted populations after 1 wk of stimulation in the absence or in the presence of rapamycin was assessed as in Fig. 3 above using PHA. Data are shown for two donors as mean values including SD.

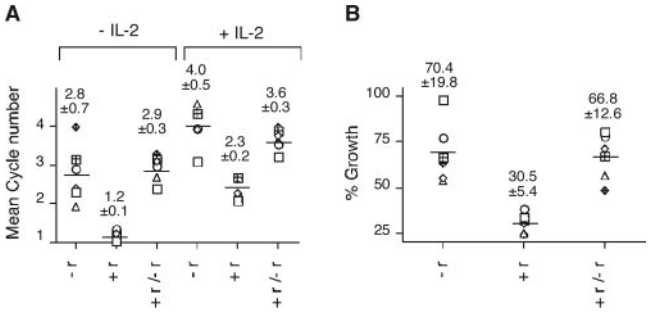

FIGURE 5. Rapamycin-induced hyporesponsiveness and suppressor functions in conventional $CD4^+$ T cells are completely reversed by restimulation in the absence of rapamycin. $CD4^+$ T cells stimulated in the presence of rapamycin and IL-2 during two stimulation cycles were restimulated during one additional week in the absence of rapamycin (+r/−r) and in the presence of IL-2. After this treatment, these populations, together with $CD4^+$ T cell control populations stimulated in the presence (+r) or in the absence (−r) of rapamycin during two stimulation cycles in the presence of IL-2, were assessed for their ability to proliferate following an additional TCR-mediated stimulation in the absence or in the presence of IL-2 (100 IU/ml) as well as for their capacity to suppress the proliferation of responder $CD4^+$ T cells in the absence of exogenously added IL-2. *A*, Cell division of CFSE-labeled $CD4^+$ T cell populations, after the indicated treatment, was assessed 5 days after stimulation in the absence of rapamycin. Mean cycle numbers of cells in the cultures are shown for six donors, as mean values including SD. *B*, Cell division of CFSE-labeled responder $CD4^+$ T cells, in the absence or in the presence of stimulated $CD4^+$ T cell populations after the indicated treatment, was assessed as in Fig. 3. Percentage of growth was calculated as detailed in *Materials and Methods*. Data are presented for six donors as mean values including SD.

expansion. Surprisingly, in vitro-stimulated naive $CD4^+$ $CD25^-$ exhibited a rather significant suppressor activity. However, in vitro-stimulated NnTregs exhibited an even stronger suppressive activity. In addition and importantly, for all populations, suppressor functions were increased after stimulation in the presence of rapamycin. By taking into account that the $CD25^+$ Treg populations represent, together, <5% of total $CD4^+$ T cells, that they proliferate less than conventional $CD4^+$ T cells, and that their growth is inhibited by rapamycin even more than that of conventional $CD4^+$ T cells, our data clearly demonstrate that rapamycin-mediated enrichment of T cells with suppressor functions in stimulated $CD4^+$ T cell cultures is not due to the selective expansion of naturally occurring $CD4^+CD25^{bright}$ Tregs present in the total $CD4^+$ T cell fraction, but, instead, to the ability of rapamycin to induce suppressor functions in the total $CD4^+$ T cell population.

Rapamycin induces a hypoproliferative state and suppressor functions in conventional $CD4^+$ T cells

A previous study from Powell et al. (22) has provided evidence that full activation of $CD4^+$ T cells in the presence of rapamycin results in the induction of a hypoproliferative state. In contrast, it has been shown that T cells stimulated in vitro using immobilized anti-CD3 mAb can inhibit the proliferation of responder T cells in vitro, and that in vivo, adoptive transfer of these T cells into recipients of allogeneic skin grafts leads to prolonged skin graft survival (23). To further address the mechanisms of the induction of suppressive activity observed upon stimulation of $CD4^+$ T cells in the presence of rapamycin, we assessed the ability of $CD4^+$ T cell populations stimulated in the absence or in the presence of rapamycin to respond to stimulation with anti-CD3/CD28 Abs in the absence or in the presence of IL-2. As expected, $CD4^+$ T cell populations stimulated in the absence of rapamycin efficiently responded to TCR-mediated stimulation both in the absence and in the presence of IL-2 (Fig. 5*A*). However, $CD4^+$ T cell populations stimulated in the presence of rapamycin proliferated poorly following TCR-mediated stimulation in the absence of IL-2. Proliferation, however, was partially restored by the presence of IL-2 (Fig. 5*A*). These data demonstrate that stimulation in the presence of rapamycin induces a hypoproliferative state in conventional $CD4^+$ T cells, similar to that constitutively exhibited by naturally occurring $CD4^+$ Treg populations, consistent with what was previously reported by Powell et al. (22). Also consistent with the data from Powell et al. in contrast to the reduced proliferative capacity of rapamycin-treated T cells, their IL-2 production following TCR-mediated stimulation was similar to that of $CD4^+$ T cells expanded in the absence of rapamycin (data not shown). To assess whether the hypoproliferative state induced by rapamycin was permanent or reversible, cells from rapamycin-treated populations were further stimulated in the absence of rapamycin for an additional week. Withdrawal of rapamycin from the cultures resulted indeed in a complete reversal of T cell hyporesponsiveness (Fig. 5*A*). Consistent with our previous data (Fig. 3), $CD4^+$ T cells stimulated in the presence of rapamycin suppressed the proliferation of responder $CD4^+$ T cells. The suppressor capacity of these cells, however, was abolished by an additional stimulation in the absence of rapamycin (Fig. 5*B*). Together, our data indicate that stimulation of conventional $CD4^+$ T cells in the presence of rapamycin results in the induction of a hypoproliferative state together with suppressor functions. This condition, however, is temporary and reversible as it is dependent upon the continuous presence of rapamycin.

Discussion

This study has addressed the effect of rapamycin on the growth and functions of in vitro-stimulated human $CD4^+$ T cells, with respect to the possible induction of regulatory/suppressor Tregs. Consistent with data recently published by Battaglia et al. (10), we observed that TCR-mediated stimulation of $CD4^+$ T cells in the presence of rapamycin and IL-2 results in highly increased suppressor functions. We could clearly show, however, that rapamycin had an inhibitory effect on the growth of both non-Treg $CD4^+$ populations and even more so on $CD4^+$ Treg populations. This effect was observed after a single in vitro stimulation and persisted after additional stimulations. This, together with the fact that naturally occurring Treg populations represent ex vivo a minority of total $CD4^+$ T cells, unambiguously demonstrates that the observed increased suppressor function in rapamycin-treated cultures is not due to the preferential expansion of naturally occurring Tregs. In contrast, we could clearly show that this phenomenon is due to rapamycin-induced suppressor functions on total $CD4^+$ T cells.

Consistent with previous findings reported by Powell et al. (22), we found that the presence of rapamycin, concomitant with TCR-mediated stimulation, induces a hypoproliferative state in $CD4^+$ T cells. The hypoproliferative state of rapamycin-treated cells is very likely in close relation with their suppressive functions. Indeed, it has been shown that T cells made unresponsive in vitro using immobilized anti-CD3 mAb vigorously suppress the proliferation of responder T cells in vitro and can in vivo, after adoptive transfer into recipients of allogeneic skin grafts, lead to prolonged survival of the graft (23). This is in line with the capacity of rapamycin-treated $CD4^+$ T cells to prevent allograft rejection in vivo (10). Interestingly, the suppression observed by in vitro-anergized $CD4^+$ T cells does not appear to be due to the release of inhibitory cytokines, but requires cell-cell contact as reported for naturally occurring Tregs (23). In addition, naturally occurring Tregs are constitutively anergic as they are selected as such in the thymus (24). The hypoproliferative state of $CD4^+$ T cells in rapamycin-treated cultures is distinct from anergy. Indeed, rapamycin-induced

hypoproliferation is also observed in the presence of costimulation, and does not inhibit costimulation-mediated IL-2 production (22). Thus, conventional $CD4^+$ T cells that are converted into Tregs by TCR-mediated stimulation in the presence of rapamycin are not anergic, but share some functional characteristics with both T cells anergized in vitro and naturally anergic cells of the thymically derived $CD4^+CD25^+$ lineage, including suppressor functions. Although the molecular bases of suppression remain to be determined, it can reasonably be expected that at least a fraction of the involved molecules will be common among these different types of suppressor cells.

Finally, it is noteworthy that $CD4^+$ T cells, which acquired suppressor functions through TCR-mediated stimulation in the presence of rapamycin, reverted into nonsuppressive cells after further stimulation and culture in the absence of the drug. The ability of rapamycin to induce profound, but completely reversible hypoproliferative state and suppressor functions in $CD4^+$ T cells is an important characteristic of this drug and may explain its ability to induce graft tolerance in the absence of long-term immunosuppression (5). However, it is noteworthy that, in light of our results, the appropriateness of using rapamycin to prepare Tregs from stimulated $CD4^+$ T cells for cellular therapy in T cell-mediated diseases, as suggested by Battaglia et al. (10), must be considered with caution. For this type of application, the use of purified autologous naturally occurring NnTregs (20) would seem more appropriate. Preclinical studies aimed at optimizing this approach are currently being conducted in our laboratory.

Disclosures

The authors have no financial conflict of interest.

References

1. Abraham, R. T., and G. J. Wiederrecht. 1996. Immunopharmacology of rapamycin. *Annu. Rev. Immunol.* 14: 483–510.
2. Vezina, C., A. Kudelski, and S. N. Sehgal. 1975. Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. *J. Antibiot.* 28: 721–726.
3. Blaha, P., S. Bigenzahn, Z. Koporc, M. Schmid, F. Langer, E. Selzer, H. Bergmeister, F. Wrba, J. Kurtz, C. Kiss, et al. 2003. The influence of immunosuppressive drugs on tolerance induction through bone marrow transplantation with costimulation blockade. *Blood* 101: 2886–2893.
4. Wiederrecht, G. J., C. J. Sabers, G. J. Brunn, M. M. Martin, F. J. Dumont, and R. T. Abraham. 1995. Mechanism of action of rapamycin: new insights into the regulation of G_1-phase progression in eukaryotic cells. *Prog. Cell Cycle Res.* 1: 53–71.
5. Kahan, B. D., and J. S. Camardo. 2001. Rapamycin: clinical results and future opportunities. *Transplantation* 72: 1181–1193.
6. Mita, M. M., A. Mita, and E. K. Rowinsky. 2003. Mammalian target of rapamycin: a new molecular target for breast cancer. *Clin. Breast Cancer* 4: 126–137.
7. Yu, K., L. Toral-Barza, C. Discafani, W. G. Zhang, J. Skotnicki, P. Frost, and J. J. Gibbons. 2001. mTOR, a novel target in breast cancer: the effect of CCI-779, an mTOR inhibitor, in preclinical models of breast cancer. *Endocr. Relat. Cancer* 8: 249–258.
8. Boulay, A., S. Zumstein-Mecker, C. Stephan, I. Beuvink, F. Zilbermann, R. Haller, S. Tobler, C. Heusser, T. O'Reilly, B. Stolz, et al. 2004. Antitumor efficacy of intermittent treatment schedules with the rapamycin derivative RAD001 correlates with prolonged inactivation of ribosomal protein S6 kinase 1 in peripheral blood mononuclear cells. *Cancer Res.* 64: 252–261.
9. Tian, L., L. Lu, Z. Yuan, J. R. Lamb, and P. K. Tam. 2004. Acceleration of apoptosis in $CD4^+CD8^+$ thymocytes by rapamycin accompanied by increased $CD4^+CD25^+$ T cells in the periphery. *Transplantation* 77: 183–189.
10. Battaglia, M., A. Stabilini, and M. G. Roncarolo. 2005. Rapamycin selectively expands $CD4^+CD25^+FoxP3^+$ regulatory T cells. *Blood* 105: 4743–4748.
11. Onizuka, S., I. Tawara, J. Shimizu, S. Sakaguchi, T. Fujita, and E. Nakayama. 1999. Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor α) monoclonal antibody. *Cancer Res.* 59: 3128–3133.
12. Belkaid, Y., C. A. Piccirillo, S. Mendez, E. M. Shevach, and D. L. Sacks. 2002. $CD4^+CD25^+$ regulatory T cells control *Leishmania major* persistence and immunity. *Nature* 420: 502–507.
13. Cohen, J. L., A. Trenado, D. Vasey, D. Klatzmann, and B. L. Salomon. 2002. $CD4^+CD25^+$ immunoregulatory T cells: new therapeutics for graft-versus-host disease. *J. Exp. Med.* 196: 401–406.
14. Bach, J. F., and J. Francois Bach. 2003. Regulatory T cells under scrutiny. *Nat. Rev. Immunol.* 3: 189–198.
15. Roncarolo, M. G., and M. K. Levings. 2000. The role of different subsets of T regulatory cells in controlling autoimmunity. *Curr. Opin. Immunol.* 12: 676–683.
16. Weiner, H. L. 2001. Induction and mechanism of action of transforming growth factor-β-secreting Th3 regulatory cells. *Immunol. Rev.* 182: 207–214.
17. Jordan, M. S., A. Boesteanu, A. J. Reed, A. L. Petrone, A. E. Holenbeck, M. A. Lerman, A. Naji, and A. J. Caton. 2001. Thymic selection of $CD4^+CD25^+$ regulatory T cells induced by an agonist self-peptide. *Nat. Immunol.* 2: 301–306.
18. Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α-chains (CD25): breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 155: 1151–1164.
19. 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.
20. Valmori, D., A. Merlo, N. E. Souleimanian, C. S. Hesdorffer, and M. Ayyoub. 2005. A peripheral circulating compartment of natural naive CD4 Tregs. *J. Clin. Invest.* 115: 1953–1962.
21. Walker, M. R., D. J. Kasprowicz, V. H. Gersuk, A. Benard, M. Van Landeghen, J. H. Buckner, and S. F. Ziegler. 2003. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human $CD4^+CD25^-$ T cells. *J. Clin. Invest.* 112: 1437–1443.
22. Powell, J. D., C. G. Lerner, and R. H. Schwartz. 1999. Inhibition of cell cycle progression by rapamycin induces T cell clonal anergy even in the presence of costimulation. *J. Immunol.* 162: 2775–2784.
23. Chai, J. G., I. Bartok, P. Chandler, S. Vendetti, A. Antoniou, J. Dyson, and R. Lechler. 1999. Anergic T cells act as suppressor cells in vitro and in vivo. *Eur. J. Immunol.* 29: 686–692.
24. Itoh, M., T. Takahashi, N. Sakaguchi, Y. Kuniyasu, J. Shimizu, F. Otsuka, and S. Sakaguchi. 1999. Thymus and autoimmunity: production of $CD25^+CD4^+$ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J. Immunol.* 162: 5317–5326.