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

Danila Valmori, Valeria Tosello, Naira E. Souleimanian, Emmanuelle Godefroy, Luigi Scotto, Yu Wang and Maha Ayyoub

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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

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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.

Ludwig Institute Clinical Trial Center, Department of Medicine, Columbia University College of Physicians and Surgeons, New York, NY 10032

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2 Address correspondence and reprint requests to Dr. Danila Valmori, Ludwig Institute Clinical Trial Center, Division of Medical Oncology, Department of Medicine, Columbia University College of Physicians and Surgeons, 650 West 168th Street, Black Building Room 20-09, New York, NY 10032; E-mail address: dv2117@columbia.edu or Dr. Maha Ayyoub, Ludwig Institute Clinical Trial Center, Division of Medical Oncology, Department of Medicine, Columbia University College of Physicians and Surgeons, 650 West 168th Street, Black Building Room 20-09, New York, NY 10032; E-mail address: msa2106@columbia.edu

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lineage in the thymus and that constitutively express CD25. Thy-
mically derived Tregs are constitutively anergic (17). The key role
of cells from this subset in the maintenance of tolerance is dem-
onstrated 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 com-
plicated by the fact that CD25 is not exclusively expressed by
Tregs, but also by nonregulatory lymphocytes, following activa-
tion. 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+CD25bright) 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
defined as natural naive (Nn) Tregs (20). NnTregs represent the naive
circulating compartment of human naturally occurring Tregs.
Due to their 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 absence 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 phenom-
enon by assessing the effect of rapamycin on the growth and dif-
ferentiation of non-Treg and Treg populations of defined differen-
tiation stages, highly purified ex vivo from circulating CD4+ T
cells, we found that the increased suppressive activity in rapamy-
cin-treated cultures is not due to the selective expansion of natur-
ally occurring Tregs, but to rapamycin-induced anergy and sup-
pressor 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 en-
riched 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 Laborato-
ries), 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
FACS aria (BD Biosciences). Data analysis was performed using the
FACS Diva 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) (eBioscience) 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 $n_f$, where $n$ is
the cycle number and $f$ is the fraction of cells that have un-
dergone $n$ 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 × 105cells/well) with suppressors at a suppressor to responder cell ratio
of 1:1, in 96-well U-bottom plates in the presence of 2 × 106/well irradiated
allogeneic CD4+CD8− T 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 sup-
pressor cells (experimental group) was compared with that in the wells
without suppressors (control group). The percentage of growth was deter-
mined at day 5 after stimulation as followings (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 (Pro-
mega), 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 CTC AGC CCA
CGG TCA T-3′ and reverse primer, 5′-CAG AAA GCA CTT GTG CAG
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: 5′-AAG GAT AGG GTG ACG GTA
GTC AAC GGA TTG G-3′TAMRA. Primer sequences for GAPDH were
as follows: 5′-CCCATCGGTATACACCAT-3′ and 5′-CCAGGCCG
CCAATACG-3′ (Applied Biosystems).

Results

Stimulation of human CD4+ T cells in the presence of
rapamycin results in increased proportions of CD25bright T cells
and in increased suppressive activity

To initially assessed the effect of rapamycin on the growth and differen-
tiation 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 stimu-
lated with plate-bound anti-CD3/CD28 Abs, APCs, and IL-2 in the
absence or in the presence of rapamycin. To follow cell divi-
sion, one part of the cells was labeled with CFSE before stimula-
tion. 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 unindividuated 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 rapa-
mycin (Fig. 1D). 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 propor-
tion 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,
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. CD4⁴+ T cells stimulated and cultured in the presence of rapamycin contained higher proportions of CD25bright T cells compared with control cultures. The increase of CD25bright 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 CD4⁴+ 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, CD4⁴+ T cells from rapamycin-treated and control cultures were cocultured with allogeneic CFSE-labeled responder CD4⁴+ T cells and stimulated with either PHA or anti-CD3/CD28 Abs. As illustrated in Fig. 3, in both experimental settings, rapamycin-treated CD4⁴+ 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 CD4⁴+ T cells with increased suppressive activity in rapamycin-treated murine cultures is due to a selective expansion of the low proportions of naturally occurring CD4⁴+ Tregs present in the total CD4⁴+ T cell populations at the beginning of the cultures. To assess the relevance of these findings in our experimental system, we stained highly enriched CD4⁴+ 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 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.

FIGURE 2. Rapamycin delays the differentiation of stimulated CD4⁺ T cells and promotes the increase of CD4⁺CD25bright 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 CD25bright T cells was determined as shown for one donor, on gated CD4⁺ T cells. D, Data are presented for six donors.
CD45RA/H11002 and CD25/H11002), and the CD45RA−CD25− population corresponding to Ag-experienced Tregs (Fig. 4A). 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. 4B, after the first cycle of stimulation, all sorted populations, with the exception of CD45RA−CD25bright 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 non-regulatory ones, although NnTregs proliferated more than Tregs (Fig. 4B). 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. 4B). 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. 4C). 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. 4D). 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 stimulation.

**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.

**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+ T cells were sorted into four populations: conventional naive (N, CD45RA+CD25−), NnTregs (CD45RA+CD25+), conventional Ag-experienced (AE, CD45RA−CD25low), and Tregs (CD45RA−CD25bright), 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.
the presence of rapamycin, we assessed the ability of CD4
absence or in the presence of IL-2. As expected, CD4
functions in conventional CD4
Rapamycin-induced hyporesponsiveness and suppressor
expansion (23). To further address the mechanisms of the induction of
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A previous study from Powell et al. (22) has provided evidence
full activation of CD4
CD4
T cells, similar to that constitutively exhibited by naturally
occuring CD4
Treg populations, consistent with what was pre-
viously 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 fur-
ther 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. 5A). Consis-
tent 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 rapa-
mycin (Fig. 5B). 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. Consis-
tent with data recently published by Battaglia et al. (10), we ob-
served that TCR-mediated stimulation of CD4
T cells in the pres-
ence 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
popula-
tions and even more so on CD4
Treg populations. This effect was
observed after a single in vitro stimulation and persisted after ad-
ditional stimulations. This, together with the fact that naturally
occuring Treg populations represent ex vivo a minority of total
CD4
T cells, unambiguously demonstrates that the observed in-
creased 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-activated
CD4
T cells does not appear to be due to the release of inhibitory
cytokines, but requires cell-cell contact as reported for naturally
occuring 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

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 re-
ipients of allogeneic skin grafts leads to prolonged skin graft sur-
vival (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 rapa-
mycin 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 re-
sponded to TCR-mediated stimulation both in the absence and in
the presence of IL-2 (Fig. 5A). However, CD4
T cell populations
stimulated in the presence of rapamycin proliferated poorly fol-
lowing TCR-mediated stimulation in the absence of IL-2. Prolif-
eration, however, was partially restored by the presence of IL-2
(Fig. 5A). 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
occuring CD4
Treg populations, consistent with what was pre-
viously reported by Powell et al. (22). Also consistent with the data
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ther stimulated in the absence of rapamycin for an additional week.
Withdrawal of rapamycin from the cultures resulted indeed in a
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functions. This condition, however, is temporary and reversible as it
is dependent upon the continuous presence of rapamycin.

Rapamycin-induced hyporesponsiveness and suppressor-functions in conventional CD4
T cells

In addition and importantly, for all populations, suppressor
functions were increased after stimulation in the presence of rapam-
ycin. By taking into account that the CD25
Treg populations repre-
sent, 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
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.
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 to nonsuppressive cells after further stimulation and culture in the absence of the drug. The ability of rapamycin to induce profound, but completely reversible hypoproliferation and culture in the absence of the drug. The ability of rapamycin to induce profound, but completely reversible hypoproliferation and culture in the absence of the drug.

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