Selective Survival of Naturally Occurring Human $CD4^+CD25^+Foxp3^+$ Regulatory T Cells Cultured with Rapamycin[1]

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Naturally occurring $CD4^+CD25^+$ regulatory T (nTreg) cells are essential for maintaining T cell tolerance to self Ags. We show that discrimination of human Treg from effector $CD4^+CD25^+$ non-nTreg cells and their selective survival and proliferation can now be achieved using rapamycin (sirolimus). Human purified $CD4^+CD25^{high}$ T cell subsets stimulated via TCR and CD28 or by IL-2 survived and expanded up to 40-fold in the presence of 1 nM rapamycin, while $CD4^+CD25^{low}$ or $CD4^+CD25^-$ T cells did not. The expanding pure populations of $CD4^+CD25^{high}$ T cells were resistant to rapamycin-accelerated apoptosis. In contrast, proliferation of $CD4^+CD25^-$ T cells was blocked by rapamycin, which induced their apoptosis. The rapamycin-expanded $CD4^+CD25^{high}$ T cell populations retained a broad TCR repertoire and, like $CD4^+$ $CD25^+$ T cells freshly obtained from the peripheral circulation, constitutively expressed CD25, Foxp3, CD62L, glucocorticoid-induced TNFR family related protein, CTLA-4, and CCR-7. The rapamycin-expanded T cells suppressed proliferation and effector functions of allogeneic or autologous $CD4^+$ and $CD8^+$ T cells in vitro. They equally suppressed Ag-specific and nonspecific responses. Our studies have defined ex vivo conditions for robust expansion of pure populations of human nTreg cells with potent suppressive activity. It is expected that the availability of this otherwise rare T cell subset for further studies will help define the molecular basis of Treg-mediated suppression in humans. ***The Journal of Immunology,*** **2007, 178: 320–329.**

A growing body of evidence suggests that regulatory T (Treg)[3] cells are capable of inhibiting most types of immune responses. They play key roles not only in the maintenance of immunological self-tolerance, but also in limiting responses to foreign Ags. Thus, Treg cells have been implicated in autoimmune diseases, organ transplantation, and infectious diseases (1–3). Importantly, Treg cells can also dampen antitumor T cell immunity and have been proposed as a potential mechanism of immune escape (4). Previously, increased proportions of Treg cells among the total $CD4^+$ T cell populations have been described in the circulation and tumor tissues of patients with different types of cancer (5–7). In ovarian cancer, accumulations of Treg cells in the tumor predicted a significant reduction in survival (8). To date, at least two types of $CD4^+$ Treg cells have been partly characterized in humans: 1) Treg type 1 and Th3 cells, which are induced in the periphery upon encountering cognate Ags and secrete IL-10 and TGF-β, respectively (9, 10) and 2) naturally occurring $CD4^+CD25^{high}Foxp3^+$ T cells (naturally occurring $CD4^+CD25^+$ Treg (nTreg) cells) which arise directly in the thymus and have the ability to suppress responses of both $CD4^+CD25^-$ and $CD8^+CD25^-$ T cells (11–14). nTreg cells express the transcription factor, forkhead box p3 (Foxp3), which has been associated with their development and suppressive function (15, 16). In addition, they express glucocorticoid-induced TNFR family related gene (GITR) and intracellular CTLA-4. More recent studies indicate that $CD4^+CD25^{high}Foxp3^+$ T cells are a heterogeneous population within $CD4^+$ T cells, endowed with different regulatory functions, including regulation of Ag-specific immune responses (17, 18). As our knowledge of Treg-mediated immunosuppression has increased, the potential for therapeutic alterations in immunity through in vivo and in vitro manipulation of Treg cell numbers and/or functions in disease holds great promise.

Currently, isolation and expansion of human nTreg cell subsets into functionally active, disease-specific T cells is, however, difficult due to the following: 1) the paucity of nTreg cells in the peripheral blood; 2) the lack of specific identity markers for nTreg cells; and 3) the absence of protocols for rapid and extensive expansion of pure nTreg cells with suppressive activity from peripheral blood. In humans, $CD4^+CD25^+$ T cells include suppressor $CD4^+CD25^{high}$ T cells as well as $CD4^+CD25^{low}$ T cells which are nonsuppressive, activated $CD4^+$ T cells. Furthermore, CTLA-4 and GITR are activation Ags and because levels of their expression vary depending on cell activation, they are not useful for discriminating nTreg from effector T cell populations. Similarly, Foxp3

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[3] Abbreviations used in this paper: Treg, regulatory T; nTreg, naturally occurring $CD4^+CD25^+$ Treg; GITR, glucocorticoid-induced TNFR family related protein; TT, tetanus toxoid; ANX V, annexin V; LN, lymph node; MFI, mean fluorescence intensity; PI, propidium iodine.

expression—though more specific for Treg—may also be up-regulated on effector cells following activation (19), and due to its intracellular localization, Foxp3 cannot be used for separation of living Treg cells. As a consequence, most current studies using $CD4^+CD25^+$ populations for analysis of the phenotype and functions of nTreg cells are not interpretable due to the presence of contaminating effector $CD4^+$ T cells, which reduce or even completely camouflage suppressive functions of nTreg cells present in T cell cultures.

It has been shown that the immunosuppressive drug rapamycin (sirolimus) is a powerful pharmacological agent which blocks intracellular signaling in response to T cell growth factors, e.g., IL-2, required for the progression of activated T cells from G_1 into S phase (20). Though rapamycin has been reported to favor $CD4^+CD25^+$ T cell-dependent immunoregulation in vitro and in vivo (21–25), the exact mechanism of its action on nTreg cells remains largely unknown. In particular, a direct effect of rapamycin on survival and proliferation of human nTreg cells has not been demonstrated.

To gain insights into the phenotypic and functional characteristics of expanded, phenotypically homogeneous, and highly suppressive populations of human $CD25^{high}$ nTreg cells, we have used rapamycin to discriminate between $CD4^+CD25^{high}Foxp3^+$ T cells with a constitutive expression of CD25 from $CD4^+CD25^+$ effector T cells and $CD4^+CD25^-$ resting T cells. In this study, we provide new evidence that in the presence of IL-2, rapamycin alone or rapamycin combined with stimulation signals delivered via TCR and CD28 is capable of fostering the selective survival and expansion of $CD4^+CD25^{high}Foxp3^+$ nTreg cells, which are endowed with potent suppressive activities and thus may serve as pure populations of human nTreg cells for future investigations and therapy.

Materials and Methods

Reagents and Abs

All Abs were purchased from BD Pharmingen except anti-GITR (R&D Systems) and Foxp3 (eBioscience). Rapamycin, LPS, PHA, PMA, ionomycin, and saponin were obtained from Sigma-Aldrich. IL-2 and IL-7 cytokines were obtained from R&D Systems. Monensin was obtained from BD Pharmingen. T cells were cultured in RPMI 1640 (Invitrogen Life Technologies) supplemented with human AB serum (8% v/v), L-glutamine (200 mM), sodium pyruvate (1%), nonessential amino acids (1%), penicillin/streptomycin (1%) (all obtained from Invitrogen Life Technologies), 2-ME (1%) (Sigma-Aldrich), and fluoroquinolon (1%) (ciproxin infusion; Bayer).

Peptides were synthesized by standard solid-phase chemistry on a multiple peptide synthesizer (Biosynthesis). The following synthetic peptides were used: tetanus toxoid$_{947–967}$ (FNNFTVSFWLRVPK) ($TT_{947–967}$), MAGE-A3$_{247–258}$ (TQHFV QENYLEY) (MAGE-A3$_{247–258}$), Melan-A/MART1$_{26–35}$ (ELAGIGILTV) (Melan-$A_{26–35}$), HIV-POL$_{476–484}$ (ILKEPVHGV) ($HIV_{476–484}$). Synthesis of PE-labeled HLA-A2/Melan-$A_{26–35}$ multimeric complexes was performed as previously described (26).

Cell isolation

PBMC were isolated from buffy coat preparations derived from the whole blood of healthy volunteers (Suisse Blood Center, Zurich, Switzerland and Central Blood Bank, Pittsburgh, PA) by density sedimentation on Ficoll-Hypaque gradients. Cells recovered from the gradient interface were washed twice in RPMI 1640 medium, counted, and immediately used for MACS and staining. Briefly, $CD4^+$ T cells were negatively selected from the total PBMC using the CD4 isolation kit (Miltenyi Biotec), yielding a population of $CD4^+$ cells with purity of 92–98%. Positive selection on anti-CD25 magnetic microbeads was then used to separate the negative fraction containing $CD4^+CD25^-$ T cells from the $CD4^+CD25^+$ T cell fraction, using the $CD4^+CD25^+$ T Regulatory Cell Isolation kit from Miltenyi Biotec. Cells were then applied to a second magnetic column, washed, and eluted again. This procedure led to the complete positive selection of $CD4^+CD25^+$ T cells (purity ≥96%), and negative depletion of $CD4^+CD25^-$ T cells, as measured by flow cytometry. Both cell subsets were used for in vitro expansion.

In vitro expansion of MACS-purified $CD4^+$ T cells

Initially, purified $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells were cultured in Terasaki plates (10 cells/well) with syngeneic irradiated (3000 rad) PBMC (20,000 cells/well), PHA (1 μg/ml), and 50 IU IL-2/ml for 7–8 days to increase their numbers. Next, aliquots of cells (150,000 cells/well) were transferred to wells of a 96-well plate and cultured with anti-CD3/anti-CD28 Ab-coated magnetic Dynabeads (CD3/CD28 beads; Dynal Biotech/Invitrogen Life Technologies) at 4×10^7 beads/ml and 1,000 IU/ml IL-2 for 7 days. Rapamycin at the final concentration of 1 nM was added to half of the cultures (Fig. 2). After 1 wk of expansion, cells were washed to remove IL-2 and the beads were removed with a magnetic particle concentrator (Miltenyi Biotec). The expanded cells were then transferred to wells of a 24-well plate and restimulated with CD3/CD28 beads and 1000 IU/ml IL-2. These cells were cultured in the presence or absence of 1 nM rapamycin for 1 wk (Fig. 2). Cells cultured in the absence of rapamycin are designated as "R0" and those cultured with 1 nM rapamycin as "R1." After every week of expansion, cells sampled for assays were washed twice, the beads were removed, and the cell number was determined by direct counting to evaluate expansion. Cell aliquots sampled from each group (at least 200,000 cells) were maintained for 48 h in culture medium and low-dose IL-2 (50 IU/ml) in the absence of CD3/CD28 beads and rapamycin before assays.

To evaluate effects of rapamycin on expansion of fresh MACS-purified T cells, $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were cultured ± rapamycin in the presence of 50 IU/ml IL-2 or PHA and irradiated syngeneic APC for 7 days and used for assays.

Semiquantitative RT-PCR for Foxp3

For RNA extraction, the RNase easy kit (Qiagen) was used according to the manufacturer's recommendation. Extracted RNA was reverse transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen Life Technologies). We performed quantitative PCR using the TaqMan ABI Prism 7000 Sequence Detection System (Applied Biosystems) on cDNA samples for Foxp3 expression normalized to the housekeeping gene 18s rRNA. The following primers for PCR were purchased from Applied Biosystems: forkhead/winged helix transcription factor (Foxp3), 5′-GCACCT TCCCAAATCCCAGT-3′ (sense) and 5′-GCAGGCAAGACAGTG GAAACC-3′ (antisense) in a 20-μl volume. Levels of 18s rRNA were quantified by using TaqMan PDAR Eukaryotic 18s Endogenous Controls (Applied Biosystems). The following cycling conditions were used: 10 min at 95°C, followed by 30 cycles of 15 s at 95°C, and 1 min at 60°C.

Surface and intracellular staining

All $CD4^+$ T cell samples were stained with anti-CD4, monocyte-discriminating Abs (anti-CD14, anti-CD32, and anti-CD116). Fresh as well as in vitro-expanded $CD4^+CD25^+$ (R0 vs R1) and $CD4^+CD25^-$ T cells (at least 200,000 cells/tube) were stained with the following mAbs for 15 min at 4°C to determine the "nTreg" surface marker profile: PE-conjugated anti-CD25, PerCP-labeled anti-CD4, allophycocyanin-conjugated anti-CD45RO, CCR7, and HLA-DR, FITC-conjugated anti- CD62L, Fas, GITR, CD45RA, and CD27. Criteria used for designating T cells as $CD4^+CD25^{high}$ were based on mean fluorescence intensity (MFI) as follows: with fresh PBMC when MFI was ≥20, and with isolated/cultured $CD4^+CD25^+$ T cells, which were highly enriched populations, when MFI was ≥500, as illustrated in Fig. 1, *A* and *B*. Appropriate isotype controls were included in all experiments.

Intracellular levels of IL-10 in fresh or in vitro-expanded R0 and R1 T cells were measured after a 16-h incubation with 25 ng/ml LPS (Sigma-Aldrich) in the presence of 1 μg/ml monensin (GolgiStop; BD Pharmingen). Intracellular staining was performed as previously described (27). Briefly, for detection of intracellular Foxp3, CTLA-4, and IL-10, samples were first incubated with mAbs against surface markers CD4 and CD25. After subsequent washes, cells were fixed with PBS containing 4% formaldehyde for 20 min at room temperature, washed once with PBS containing 0.5% BSA and 2 nM EDTA, permeabilized with PBS containing 0.5% (w/v) BSA and 0.2% (v/v) saponin, and stained with anti-CTLA-4-allophycocyanin, anti-Foxp3-FITC-labeled or anti-IL-10-PE-labeled mAb for 30 min at room temperature. Cells were further washed twice with PBS containing 0.5% BSA and 0.2% saponin, resuspended in FACS flow solution, and immediately analyzed by flow cytometry. Appropriate isotype controls were used in all experiments.

$CD4^+CD25^+$ and $CD4^+CD25^-$ T cells obtained by MACS isolation and R0 as well as R1 cells were assessed for annexin V (ANX V) binding. Samples were stained with FITC-conjugated ANX V (Molecular Probes/

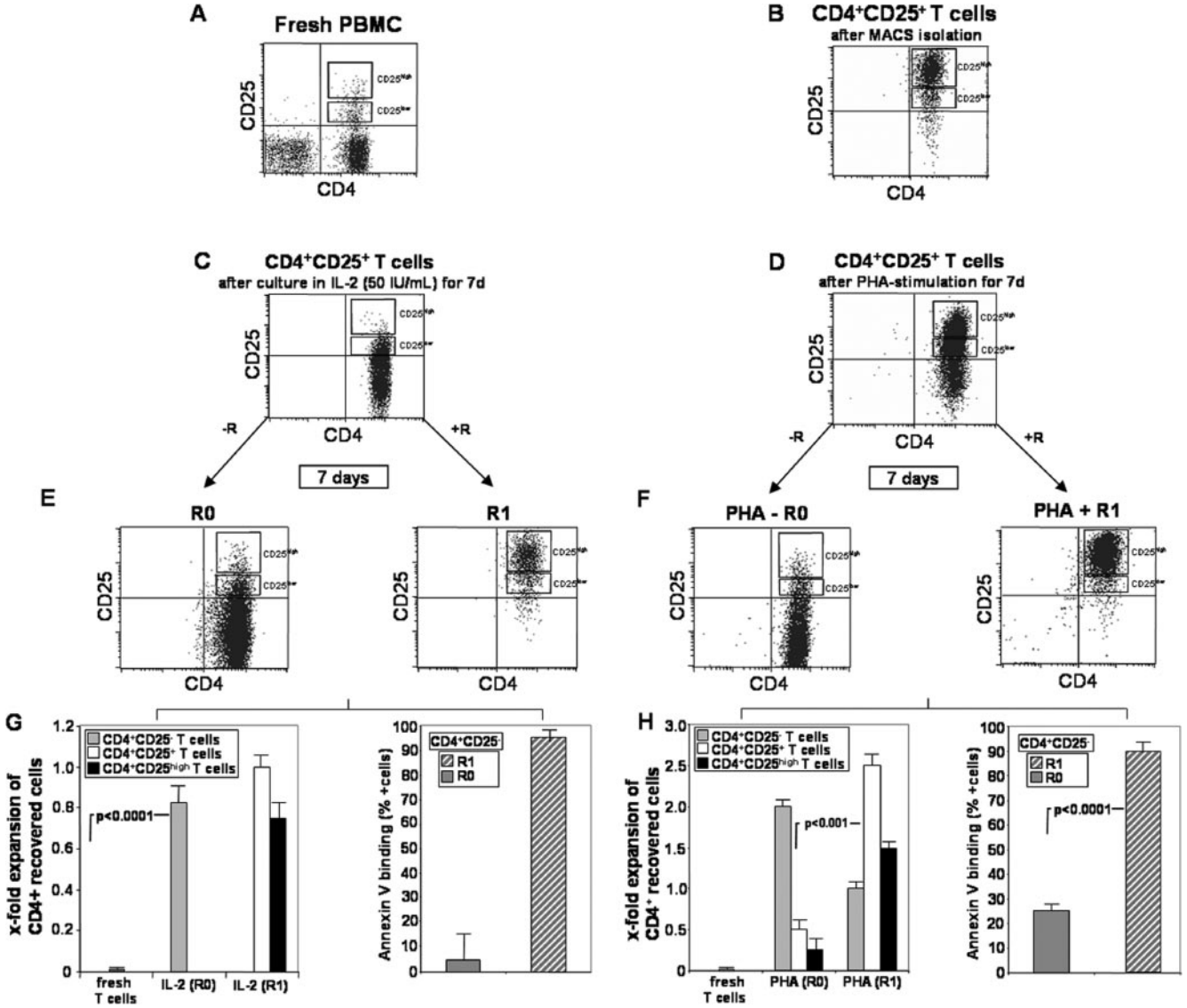

FIGURE 1. CD25 expression on fresh, activated, and cultured $CD4^+CD25^+$ T cells before and after rapamycin exposure. *A*, Expression of CD4 and CD25 on freshly isolated PBMC. *B*, Expression of CD4 and CD25 on MACS-purified human T lymphocytes. *C*, MACS-purified T cells after 1 wk of culture in the presence of 50 IU/ml IL-2. *D*, MACS-purified T cells after a 1-wk expansion in the presence of PHA (1 μg/ml) and irradiated APC in the presence of 50 IU/ml IL-2. *E* and *F*, Expression of CD4 and CD25 on MACS-purified T cells after a further 1-wk expansion (in the presence of 50 IU/ml IL-2 or PHA and irradiated feeders, respectively, in the absence (R0) or presence of 1 nM rapamycin (R1)). In R0 cultures, the mean $\pm$ SD values were as follows: 2.5 $\pm$ 7% $CD25^{high}$ cells, 23 $\pm$ 9% $CD25^{low}$ cells, and 60 $\pm$ 7.1% $CD25^-$ cells (*E* and *F*, *left*). In contrast, in R1 cultures, mainly $CD25^{high}$ T cells (95 $\pm$ 9.6%), (*E* and *F*, *right*) were present. *G* and *H*, Expansion rates and ANX V binding to MACS-purified $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells cultured in the presence (R1) or absence (R0) of rapamycin. The expansion rates of $CD4^+CD25^-$ (▨), $CD4^+CD25^+$ (□), and $CD4^+CD25^{high}$ (■) cells in R0 and R1 T cells after a 1-wk expansion in 50 IU/ml IL-2 or in the presence of PHA and irradiated feeders are shown in *G* and *H*, *left*. Expansion rates are calculated based on the ratio of absolute cell counts after expansion vs absolute counts after MACS purification; percentages of $CD4^+CD25^-$ ANX V^+ cells in R0 (▨) and R1 (▨) cultures after 1 wk of expansion in 50 IU/ml IL-2 or in the presence of PHA and irradiated feeders are shown in *G* and *H*, *right*. *A–H*, The results of experiments performed with cells obtained from five healthy individuals. The data are representative flow cytometry dot plots (*A–F*) or means $\pm$ SD of five experiments.

Invitrogen Life Technologies) and propidium iodide (PI; Molecular Probes), according to the instructions provided by the distributor.

Flow cytometry

Flow cytometry was performed using a FACScan flow cytometer (BD Biosciences) equipped with CellQuest software (BD Biosciences). The acquisition and analysis gates were restricted to the lymphocyte gate, as determined by characteristic forward and side scatter properties of lymphocytes. Analysis gates were restricted to the $CD3^+CD4^+$, $CD4^+CD25^+$, and $CD4^+CD25^{high}$ T cell subsets. Usually, 1×10^5 lymphocytes were collected for analysis. For ANX V-stained samples the acquisition gates were restricted to the live (PI^-) as well as the dead cell (PI^+) populations present in each sample. Six parameters (forward scatter, side scatter, and four fluorescence channels) were used for list mode data analysis.

T cell repertoire diversity

TCRV β-chain repertoire of fresh as well as in vitro-expanded R0 and R1 cells was determined by staining with a panel of Vβ chain-specific Abs (BD Pharmingen), followed by flow cytometry analysis.

Generation of Ag-specific $CD4^+25^-$ and $CD8^+25^-$ T cells

$CD4^+$ and $CD8^+$ T cells enriched from PBMC using a miniMACS device were stimulated with HLA-A*0201-restricted Melan-A/MART-1_{26-35}- and HLA-DP*0401-restricted $TT_{947-967}$ peptides. To generate peptide-specific

T cells, peptide-pulsed irradiated syngeneic APCs were cocultured with $CD4^+$ or $CD8^+$ T cells in the presence of IL-2 (25 IU/ml) and IL-7 (10 ng/ml) as described (27). To select for peptide-specific T cells in these cultures, flow cytometry was used to sort T cells stained by multimers or T cell-secreting cytokines in response to peptide stimulation. The isolated T cells were cloned and cultured as previously described (27).

Suppression of proliferation and cytokine secretion

Initially, suppression of proliferation mediated by MACS-purified $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells before and after exposure to rapamycin was analyzed using an allogeneic system. Suppressive functions of these cells immediately after isolation, after 1 wk expansion (in the presence of 50 IU IL-2/ml or PHA and irradiated APC) or after 3 wk expansion (in the presence of CD3/CD28 beads and 1000 IU IL-2/ml) cocultured with fresh allogeneic responder $CD4^+CD25^-$ or $CD8^+CD25^-$ T cells was determined. For this purpose, the responder T cells were labeled with 0.5 μM CFSE (Molecular Probes) and stimulated with OKT3 (1 μg/ml) (American Type Culture Collection) and soluble anti-CD28 Ab (1 μg/ml; Miltenyi Biotec). Suppressor cells, either $CD4^+CD25^+$ or $CD4^+CD25^-$ cell fractions, were added at the suppressor:responder ratio of 1:1. Cells were incubated for 5 days in a CO_2 incubator at 37°C.

Next, Ag-specific suppression mediated by $CD4^+CD25^+$ R0 or R1 T cells that were expanded for 3 wk was analyzed in an autologous in vitro system. $CD4^+CD25^-$ $TT_{947–967}$ peptide-specific T cell line ZH-P1 and $CD8^+CD25^-$ Melan-A/$MART1_{27–35}$ peptide-specific T cell clone ZH-P7/2 were labeled with 0.5 μM CFSE (Molecular Probes) and incubated with either R0 or R1 T cells at the 1:1 ratio. Peptide-specific responder T cells were stimulated with the relevant peptide-pulsed (2 μg/ml) APC (autologous PBMCs) or with PHA (1 μg/ml).

All CFSE data were analyzed using the ModFit software provided by Verity Software House. The percentages of suppression were calculated based on the proliferation index of responder cells alone compared with the proliferation index of cultures containing responders and Tregs. The program determines the percent of cells within each peak and the sum of all peaks in the control culture is taken as 100% of proliferation and 0% of suppression.

For IFN-γ ELISPOT analysis, the $CD4^+CD25^-$ $TT_{947–967}$ peptide-specific T cell line ZH-P1 and $CD8^+CD25^-$ Melan-$A_{27–35}$ peptide-specific T cell clone ZH-P7/2 were stimulated either with the relevant peptide ($TT_{947–967}$, Melan-A/$MART1_{26–35}$, respectively) or irrelevant peptides (MAGE-$A3_{247–258}$, HIV-$POL_{476–484}$, respectively) using HLA-DP*0401$^+$ and HLA-A*0201$^+$ target cells, respectively. Twenty-four-hour ELISPOT assays were performed as previously described (27). Maximum IFN-γ release was determined by stimulation of responder cells with PMA/ionomycin.

Statistical analysis

Differences between groups were assessed using the Student *t* test. Values of p were two tailed. Values of $p < 0.05$ were considered significant.

Results

Purification of $CD4^+CD25^+$ T cells obtained from PBMC of normal donors

We first determined that $CD4^+CD25^+$ T cells comprised 2–5% of $CD4^+$ T cells in the peripheral blood of 10 healthy individuals analyzed. The $CD25^{high}$ T cells defined as described in *Materials and Methods* represented 0.5–1% of the total $CD4^+$ T cell population, while the $CD25^{low}$ T cells accounted for 2.5% of $CD4^+$ T cells (Fig. 1*A*). Next, $CD4^+CD25^+$ T cells were isolated using MACS on anti-CD4/anti-CD25 beads. The purity of positively selected $CD4^+CD25^+$ T cells was >96%. After MACS isolation, the $CD4^+CD25^+$ cell fraction contained both $CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ cells (Fig. 1*B*).

Culture system for expansion of $CD4^+CD25^+$ T cells

Fig. 2 is a schema designed for expansion of isolated $CD4^+$ $CD25^+$ human T cells. It is based on our initial finding that these cells plated at the usual concentrations of $\sim 10^5$ cells/well in 96-well plates failed to proliferate even when IL-2, PHA, and APCs were added. We determined that plating $CD4^+CD25^+$ T cells at 10 or fewer cells per well in Terasaki plates allowed for their initial expansion to 15–20 $\times$ 10^3 cells/well and their transfer

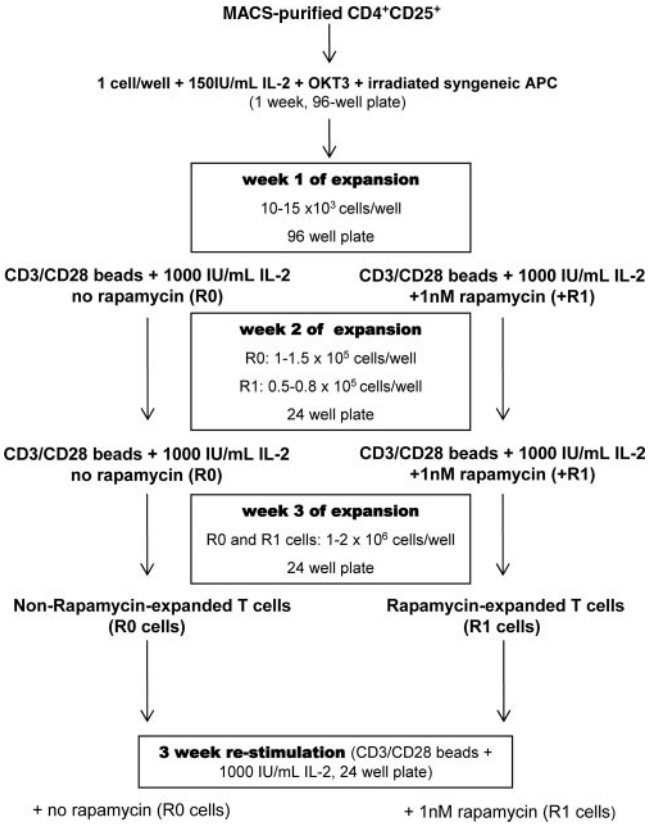

FIGURE 2. A schema for the expansion protocol of purified $CD4^+CD25^+$ T cells exposed to rapamycin. The numbers of R0 or R1 cells per well on week 1, 2, or 3 of expansion are given in the respective boxes.

to wells of 96-well plates 7 days later. To obtain sufficient cell numbers for subsequent culture ± rapamycin, $CD4^+CD25^+$ T cells remained in wells of a 96-well plate for 1 wk, yielding $\sim$100–150 $\times$ 10^3 cells. At this point, the cells were divided into parallel R0 and R1 cultures. Viable cell counts performed at each transfer showed that the fold expansion was 50- to 100-fold for R0 as well as for R1 cultures at the end of the entire 3-wk expansion period.

Phenotypic changes in cultured $CD4^+CD25^+$ T cells

The MACS-isolated cells were plated in the presence of 50 IU of IL-2 or in PHA and autologous feeder cells and assessed for phenotype after 1 wk of culture. In the presence of IL-2 alone, $CD4^+$ T cells down-regulated surface expression of CD25 (Fig. 1*C*). In contrast, most $CD4^+$ T cells stimulated with PHA and autologous feeder cells retained CD25 expression (Fig. 1*D*). These cultures were next incubated in the presence (R1) or absence (R0) of 1 nM rapamycin for 7 days. In IL-2 or PHA-containing R0 cultures, few $CD4^+$ T cells were $CD4^+CD25^{high}$ and most were $CD25^-$ (Fig. 1, *E* and *F*, *left*). In contrast, the parallel R1 cultures were enriched in $CD4^+CD25^{high}$ T cells (Fig. 1, *E* and *F*, *right*). The viable cell counts and ANX V-binding assays performed on day 7 indicated that most $CD4^+CD25^-$ T cells, which rapidly outgrow in the R0 cultures, are not sensitive to apoptosis (Fig. 1, *G* and *H*). In contrast, the R1 cultures largely contained proliferating $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells, representing a minority population, were highly sensitive to apoptosis (Fig. 1, *G* and *H*). These data are consistent with the preferential expansion of $CD4^+$ $CD25^+$ T cells accompanied by the significant inhibition of $CD4^+$ $CD25^-$ T cell expansion due to a high frequency of ANX V-binding cells in the R1 cultures (Fig. 1, *G* and *H*).

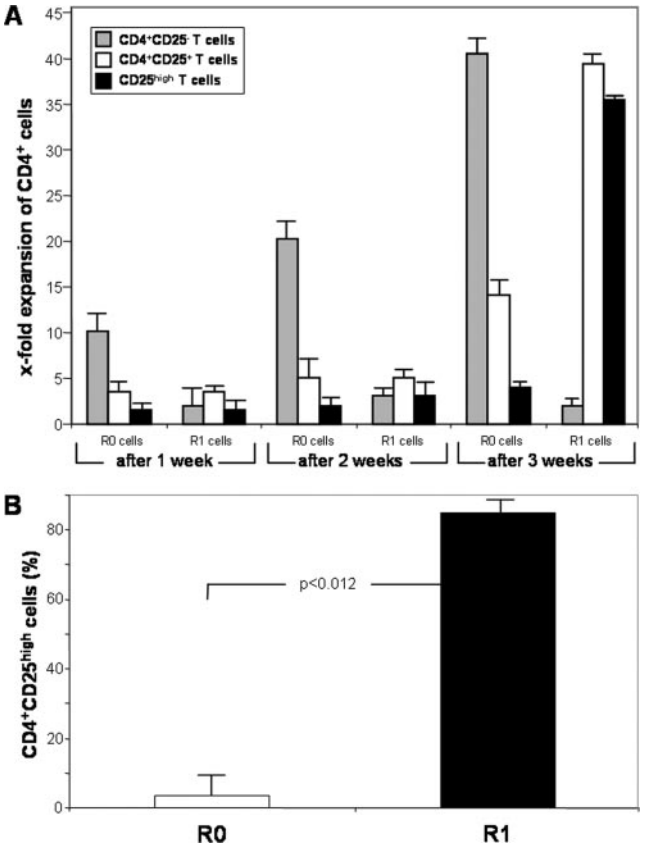

FIGURE 3. Expansion rates of in vitro-expanded human $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells in the presence or absence of rapamycin. *A*, Expansion rate of $CD4^+CD25^-$ (▨), $CD4^+CD25^+$ (□), and $CD4^+CD25^{high}$ (■) cells in the presence (R1) or absence (R0) of rapamycin with CD3/CD28 beads and 1000 IU/ml IL-2 throughout a 3-wk expansion period was assessed at weekly intervals. The expansion rate reflects the ratio of absolute cell counts after expansion vs that after MACS purification. *B*, The percentage of $CD4^+CD25^{high}$ T cells in R0 and R1 cultures after a 3-wk culture. The data are means ± SD of experiments performed with cells of five healthy individuals.

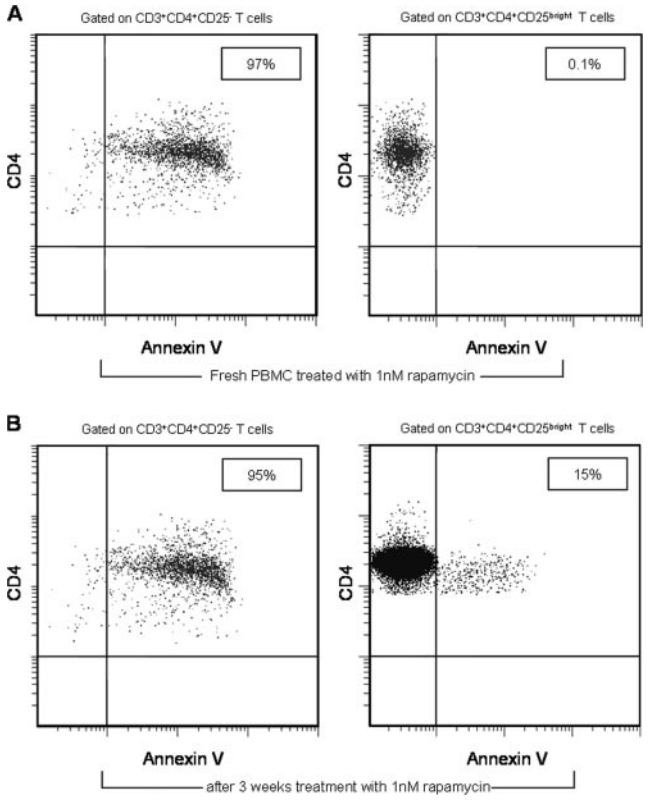

FIGURE 4. ANX V binding to fresh or cultured $CD4^+$ T cell subsets ± rapamycin. *A*, Percentages of ANX V^+ $CD4^+CD25^-$ and $CD4^+CD25^+$ cells in fresh PBMC after exposure of the cells to rapamycin for (48 h). *B*, R1 and R0 T cells were stained with ANX V and assessed by FACS analysis. In all cases, gates were set on $CD4^+CD3^+$ T cells. The data are representative dot plots from experiments performed with cells of five normal individuals.

These initial experiments indicated that rapamycin-treated $CD4^+CD25^+$ cells could expand up to 1- to 2.5-fold in cultures supplemented with IL-2 or PHA and autologous APC. The optimal rapamycin concentration for expansion of nTreg cells was next determined. In contrast to experiments performed previously in mice (25), doses of rapamycin ≥10 nM considerably decreased proliferation of MACS-purified $CD4^+CD25^+$ T cells (data not shown). Conversely, lower doses of rapamycin (≤1 nM) together with CD3/CD28 beads and 1000 IU/ml IL-2 showed no difference in expansion rates for MACS-purified $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells compared with expansion cultures without rapamycin (data not shown). A rapamycin concentration of 1 nM was determined to be optimal for expansion of $CD4^+CD25^+$ T cells and used for all additional experiments.

Fig. 3*A* illustrates expansion of $CD4^+CD25^-$, $CD4^+CD25^+$, and $CD4^+CD25^{high}$ T cell populations in parallel R0 and R1 cultures during a 3-wk expansion period. The cultures were monitored weekly for cell numbers and the percentage of $CD4^+CD25^{high}$ T cells. MACS-purified $CD4^+CD25^-$ T cells proliferated readily in the absence of rapamycin (R0), reaching considerable fold expansion by week 3. However, $CD4^+CD25^+$ as well as $CD4^+CD25^{high}$ T cells were a minor component of R0 cultures. In contrast, while expansion of $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells was delayed in the presence of rapamycin (R1), by week 3, vigorous proliferation of $CD4^+CD25^+$ and $CD4^+CD25^{high}$ T cells occurred, leading to their 50- to 100-fold expansion. $CD4^+CD25^-$ T cells were not proliferating in R1 cultures (Fig. 3*A*), while a significant enrichment in $CD4^+CD25^{high}$ T cells was routinely observed (Fig. 3*B*).

ANX V binding to MACS-purified $CD4^+CD25^+$ T cells exposed to rapamycin

Previous experiments showed that rapamycin blocked proliferation of $CD4^+CD25^-$ T cells (25) and our initial data were in agreement with this possibility. To analyze whether rapamycin selectively induces apoptosis of $CD4^+CD25^-$ T cells, we examined ANX V binding to fresh and cultured $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells after their exposure to rapamycin. As shown in Fig. 4*A*, in normal fresh PBMCs exposed to rapamycin for 48 h, the $CD4^+CD25^+$ T cells bind little ANX V (0.1 ± 1.2%). In contrast, the $CD4^+CD25^-$ T cell subset showed significantly higher ($p \leq 0.00001$) percentages of ANX V^+ cells (97 ± 12%). Thus, fresh $CD4^+CD25^+$ nTreg are resistant, while $CD4^+CD25^-$ T cells are sensitive to rapamycin-induced apoptosis. Also, a significantly higher percentage of ANX V^+ cells was observed in week 3 R1 cultures of $CD4^+CD25^-$ T cells (80 ± 13 vs 20 ± 15%) than in R0 cultures of $CD4^+CD25^-$ T cells ($p < 0.0001$), as shown in Fig. 4*B* for a representative culture. In contrast, ANX V binding to $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell subset was similar when these cells were cultured without rapamycin (23 ± 14 vs 20 ± 15%; $p < 0.44$; data not shown). Our results are consistent with the conclusion that rapamycin blocks proliferation of $CD4^+CD25^-$ T cells by inducing their apoptosis, while $CD4^+CD25^+$ Treg are resistant to apoptosis and continue to proliferate in the presence of 1 nM rapamycin.

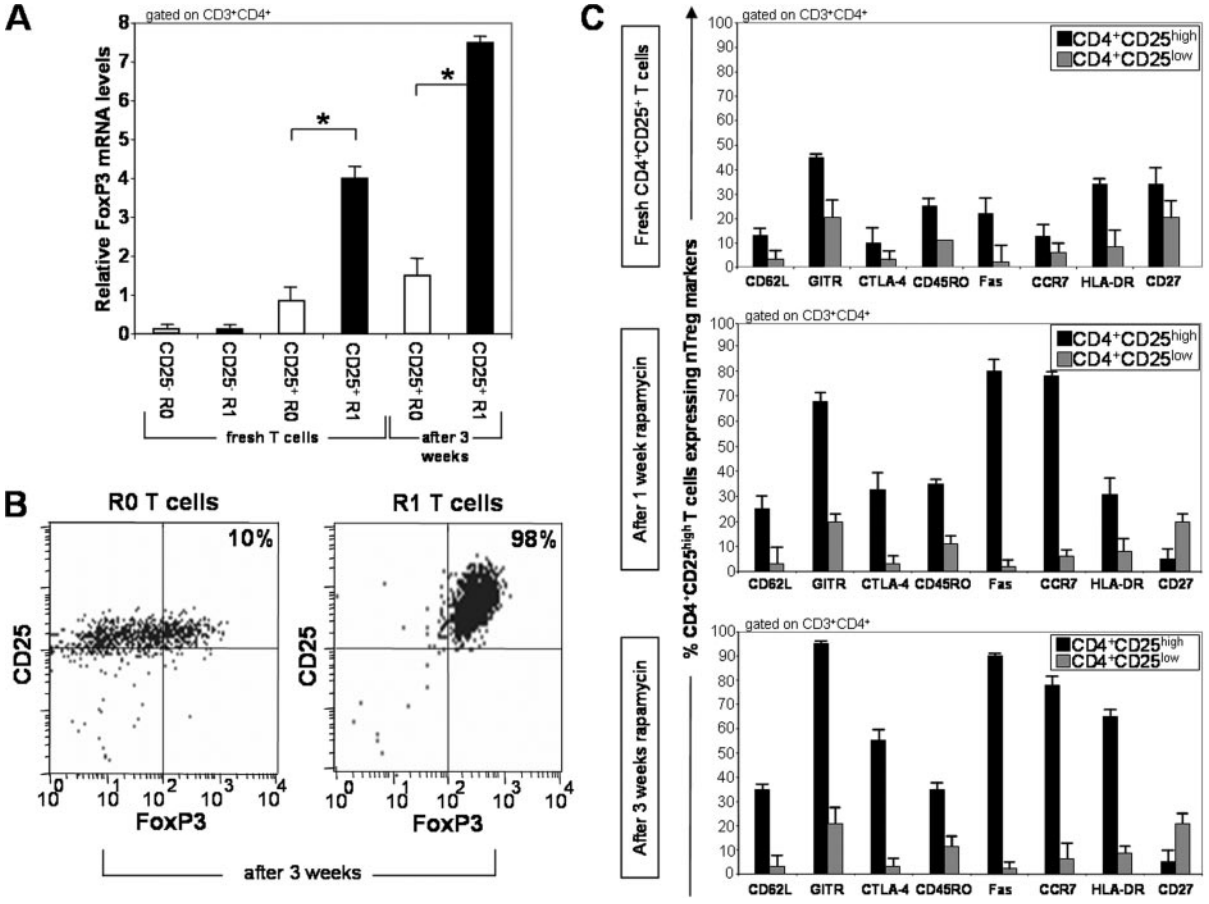

FIGURE 5. Phenotypic characterization of fresh, activated, and in vitro-expanded $CD4^+CD25^+$ T cells ± rapamycin. *A*, Relative levels of mRNA Foxp3 before and after rapamycin exposure were determined by real-time quantitative RT-PCR in fresh MACS-purified $CD4^+CD25^-$, fresh MACS-purified $CD4^+CD25^+$ T cells, and in MACS purified cells in R0 and R1 cultures after 3 wk of expansion. ∗, Significant differences ($p < 0.044$) between R0- and R1-treated cells or cultures. *B*, Flow cytometry of $CD4^+CD25^{high}$ T cells in R1 and R0 cultures after 3 wk of expansion T cells were stained with Foxp3 mAb and assessed by FACS analysis. *C*, Flow cytometry for expression of various markers on fresh $CD4^+CD25^+$ T cells and R1 T and R0 T cells after 1 and 3 wk of culture. Cells were stained with mAb against CD62L, CD27, GITR, CTLA-4, CD45RO, Fas, CCR7, and HLA-DR, and evaluated by multiparameter FACS analysis. Gates were set on $CD4^+CD25^{high}$ cells (■) or $CD4^+CD25^{low}$ cells (▨). The data are mean percentages of positive cells ± SD. Experiments were performed with fresh and cultured cells obtained from five normal individuals.

Phenotypic characteristics of MACS-purified $CD4^+CD25^+$ T cells expanded by rapamycin

Foxp3 has been considered to be a major phenotypic marker of Treg. Therefore, its expression was determined in fresh $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells as well as after 3 wk of expansion in R0 and R1 cultures. Initially, mRNA for Foxp3 was measured, using Foxp3 specific real-time quantitative PCR (Fig. 5*A*). Later, intracellular flow cytometry analysis of cells stained with Foxp3-specific Abs was used (Fig. 5*B*). Neither fresh $CD4^+CD25^-$ R0 T cells nor fresh $CD4^+CD25^-$ R1 T cells expressed Foxp3 mRNA. In contrast, $CD4^+CD25^+$ T cells before and after exposure to rapamycin expressed Foxp3 mRNA (Fig. 5*A*), and $CD4^+CD25^+$ R1 T cells expressed significantly higher levels of Foxp3 mRNA ($p < 0.044$) compared with $CD4^+CD25^+$ R0 T cells (Fig. 5*A*). We have also studied Foxp3 protein expression by fresh and rapamycin-expanded $CD4^+CD25^+$ T cells by flow cytometry. As shown in Fig. 5*B*, $CD4^+CD25^+$ T cells in 3-wk R0 cultures contained 10 ± 6.5% of $Foxp3^+$ cells. In contrast, $CD4^+CD25^+$ T cells in 3-wk R1 cultures were all Foxp3 positive (98 ± 2.5%).

To establish the phenotype of T cells expanded in the presence of rapamycin relative to that of fresh $CD4^+CD25^{high}$ T cells, multicolor flow cytometry was performed. First, the phenotype of fresh PBMC before exposure to rapamycin was determined. The $CD4^+CD25^{high}$ T cell subset in fresh PBMC expressed higher levels of CD62L, GITR, CTLA-4, CD45RO, Fas, CCR7, and HLA-DR markers compared with the $CD4^+CD25^{low}$ subset or total $CD4^+CD25^+$ T cells (Fig. 5*C*). R1 cells after 1 as well as 3 wk of expansion expressed significantly higher levels of the same markers present on the $CD4^+CD25^{high}$ subset in fresh PBMC (Fig. 5*C*; $p < 0.05$). In R1 cultures, $CD4^+CD25^{high}$ cells, which represent the proliferating and expanded subset, are $CD45RO^+$ (~40%), $CCR7^+$ (~80%), and $CD27^-$ (<10%), a phenotype resembling the central memory phenotype (28). The expression of all "nTreg" markers was higher on $CD4^+CD25^+$ T cells after 3 wk of expansion as compared with nTreg after 1 wk of expansion; however, the difference was not statistically significant ($p \leq 0.65$) (Fig. 5*C*). No significant difference in expression of the above-listed markers was observed in R1 cells expanded with <1 nM rapamycin vs R0 cells (data not shown).

Suppressor functions of MACS-purified $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells expanded by rapamycin

To evaluate suppressive activity of R0 and R1 T cells after MACS isolation and 1 wk of expansion, cocultures (in the presence of IL-2 or PHA and irradiated APC) were set up with CFSE-labeled

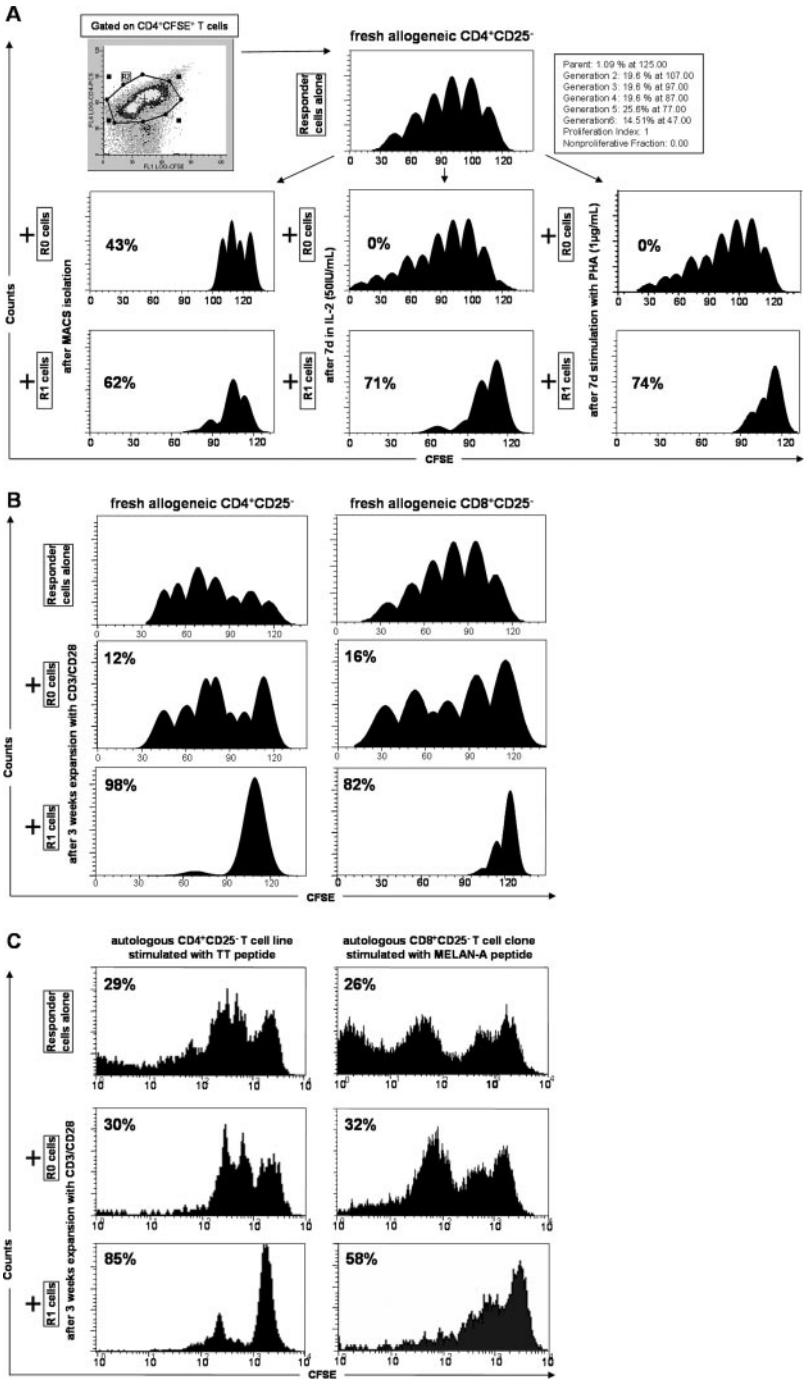

FIGURE 6. Suppression of proliferation by fresh or cultured human $CD4^+CD25^+$ T cells ± rapamycin. *A*, Freshly MACS-purified, $CD4^+CD25^-$ T cells were labeled with CFSE and stimulated with OKT3 (1 μg/ml) and anti-CD28 mAb (1 μg/ml) in the presence of 50 IU/ml IL-2. Fresh allogeneic MACS-purified $CD4^+CD25^+$ T cells or the same expanded cells from R0 and R1 cultures (7 days in 50 IU/ml IL-2 or 7 days in PHA and irradiated APC) were added at the start of the culture (ratio 1:1). Cell division represented by the dilution of CFSE was analyzed using the ModFit software as described in *Materials and Methods*. The *inset* next to control culture (responder cells alone) shows the percentages of cells in each peak after 5 days of proliferation. Histograms show CFSE intensity (*x*-axis) and the number of events (*y*-axis). The acquisition gates were restricted to the lymphocyte gate, as determined by characteristic forward and side scatter properties of lymphocytes. Analysis gates on the ModFit program were restricted to the $CD3^+CD4^+$ and $CD4^+CFSE^+$ T cell subsets, as shown on the *left*. The results of one representative experiment of five performed are shown. The percentages of proliferation inhibition are indicated in each panel. *B*, Freshly MACS-purified, $CD4^+CD25^-$, or $CD8^+CD25^-$ T cells were labeled with CFSE and stimulated with OKT3 (1 μg/ml) and anti-CD28 mAb (1 μg/ml) in the presence of 50 IU/ml IL-2. Allogeneic T cells from R0 and R1 cultures (after 3 wk of expansion with CD3/CD28 beads and 1000 IU/ml IL-2) were added at the start of the culture (ratio 1:1) and flow cytometry was performed on day 5. Note profound inhibition of proliferation by R1 but not R0 T cells. A representative experiment of five performed with PBMC of different normal donor is shown. *C*, A suppression of proliferation by R0 or R1 T cells in an autologous system. $CD4^+CD25^-$ T cell line (5×10^4 cells) (*left*) and $CD8^+CD25^-$ T cell clone (5×10^4 cells) (*right*) were labeled with CFSE and stimulated with $TT_{947–967}$ or $MELANA_{26–36}$ peptides and irradiated autologous PBMC (2.5×10^5) in the presence of IL-2. Autologous R1 and R0 T cells (after 3 wk of expansion with CD3/CD28 beads and 1000 IU/ml IL-2) were added at the start of the culture (ratio 1:1) and results were analyzed on day 5 of culture. One representative experiment of three performed with PBMC of the same ($HLA^-A2^+DR4^+$) donor is shown.

fresh allogeneic $CD4^+CD25^-$ T cells (ratio 1:1) responding to OKT3 and anti-CD28 Ab (Fig. 6*A*). The mean ± SD percentage of inhibition was 43 ± 2.5% for fresh $CD4^+CD25^+$ T cells obtained after MACS isolation from healthy donors. No suppression was observed with $CD4^+CD25^+$ T cells after 1 wk of expansion in IL-2 alone or with $CD4^+CD25^+$ T cells after 1 wk of expansion with PHA and irradiated APC (Fig. 6*A*). These cells tended to lose CD25 expression (see Fig. 1, *C* and *D*) and suppressor functions, possibly due to a rapid outgrowth of $CD4^+CD25^-$ populations (see Fig. 3*A*). In contrast, as seen in Fig. 6*A*, the mean ± SD percentages of inhibition obtained with $CD4^+CD25^+$ T cells from R1 cultures were: 62 ± 7.2% (fresh), 71 ± 3.5% (at 1 wk + IL-2), and 74 ± 6.5% (at 1 wk plus PHA/APC), respectively.

Furthermore, we analyzed the suppressive activity of R0 and R1 T cell cultures after 3 wk of expansion with CD3/CD28 beads and 1000 IU/ml IL-2 on proliferation of fresh allogeneic $CD4^+CD25^-$ and $CD8^+CD25^-$ T cells (ratio 1:1) responding to OKT3 and anti-CD28 Ab, using CFSE assays (Fig. 6*B*). The mean ± SD percentage of inhibition obtained with cells of five healthy donors was 82 ± 4.5 and 98 ± 2.35%, respectively (Fig. 6*B*), when R1 cells were tittered in at the 1:1 ratio. Much lower levels of suppression were observed (12 ± 4.5 and 16 ± 6.7%, respectively) when R0 T cells were added to the cocultures (Fig. 6*B*).

To study Ag-specific suppression in an autologous in vitro system, syngeneic R0 and R1 T cells after 3 wk of expansion with CD3/CD28 beads and 1000 IU/ml IL-2 were added at 1:1 ratios to $CD4^+CD25^-$ T cell line ZH-P1 and $CD8^+CD25^-$ T cell clone ZH-7/2, which are specific for $TT_{947–967}$ peptide and Melan-A/$MART1_{26–35}$ peptide, respectively. For this purpose, the specific T cells were stimulated with either 1) PHA or 2) APC pulsed with

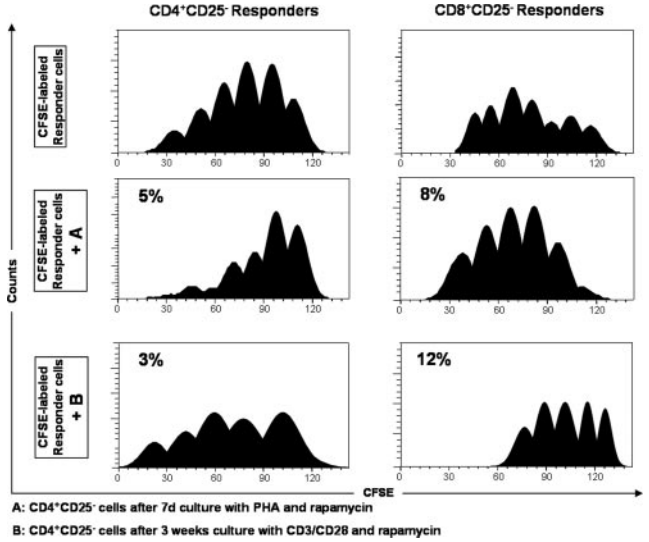

FIGURE 7. Incubation of purified $CD4^+CD25^-$ T cells in the presence or absence of rapamycin does not induce Treg. Freshly MACS-purified $CD4^+CD25^-$ or $CD8^+CD25^-$ T cells were labeled with CFSE and cultured with OKT (1 μg/ml) and anti-CD28 mAb (1 μg/ml) in the presence of IL-2 for 5 days. Allogeneic $CD4^+CD25^-$ T cells were first cultured in the presence of PHA and irradiated APC for 7 days or CD3/CD28 beads and 1000 IU/ml IL-2 for 3 wk ± rapamycin. Next, these cells were added at the 1:1 ratio to the CFSE-labeled responder cells and the cocultures incubated for 5 days. The observed percentage of suppression in these cocultures was very low (5–12%). One representative experiment (for rapamycin-expanded $CD4^+CD25^-$ cells) of five performed is shown.

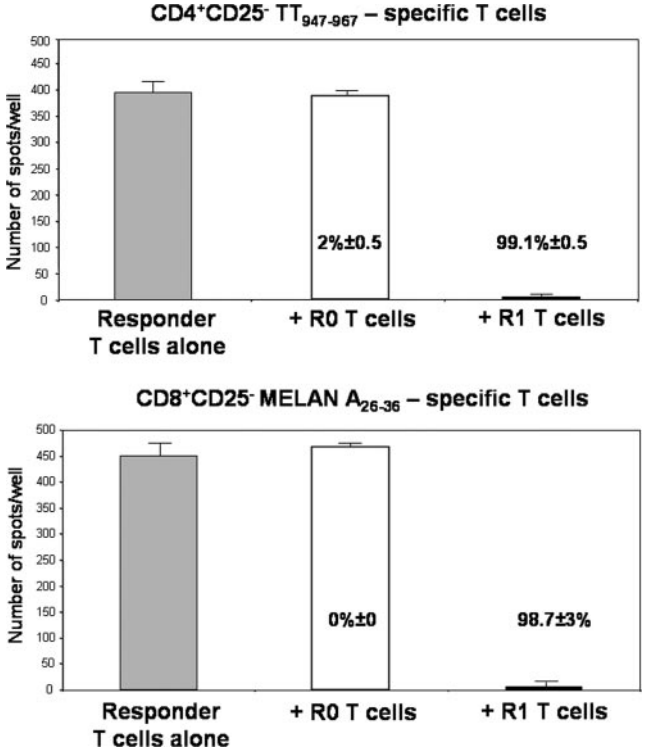

FIGURE 8. Suppression of IFN-γ secretion by purified human $CD4^+CD25^+$ T cells cultured ± rapamycin. *Top*, Autologous R1 and R0 T cells (after 3 wk of expansion with CD3/CD28 beads and 1000 IU/ml IL-2) were coincubated at the 1:1 ratio with $CD4^+CD25^-$ $TT_{947-967}$ peptide-specific polyclonal T cell line ZH-P1 for 16 h. IFN-γ release was analyzed in an IFN-γ ELISPOT assay using HLA-DP*0401-positive B-LCL cell lines pulsed with a relevant ($TT_{947-967}$) peptide or an irrelevant ($Mage\text{-}A3_{247-258}$) peptide as stimulators (*top*). *Bottom*, Autologous R1 and R0 T cells (after 3 wk of expansion with CD3/CD28 beads and 1000 IU/ml IL-2) were coincubated at the 1:1 ratio with $CD8^+CD25^-$ $Melan\text{-}A_{26-35}$-specific T cell clone ZH-P7/2 for 16 h. IFN-γ release was analyzed in an IFN-γ ELISPOT assay using HLA-A*0201-positive B-LCL cell lines pulsed with a relevant peptide ($Melan\text{-}A_{26-35}$) or an irrelevant ($HIV\text{-}POL_{476-484}$) peptide as stimulators (*bottom*). The data are numbers of spots/5 × 10^3 cells/well in 24-h ELISPOT assays. The percentages of inhibition in the number of IFN-γ-secreting positive cells by the R0 and R1 T cells are indicated in *top* and *bottom*. The data are from one representative experiment of three performed with PBMC of one HLA-DP*0401$^+$ and HLA-A*0201$^+$ donor.

the cognate peptides $TT_{947-967}$ and Melan-A/$MART1_{26-35}$, respectively. After 5 days, specific $CD4^+$ and $CD8^+$ T cell clones proliferated vigorously in response to cognate peptides in the absence of R1-expanded cells (Fig. 6*C*). However, proliferation of $CD4^+$ and $CD8^+CD25^-$ T cells after peptide stimulation was strongly inhibited upon coculture with syngeneic R1 T cells (Fig. 6*C*) but not syngeneic R0 T cells. The mean ± SD percentage of inhibition obtained with cells of the same donor in three experiments was 85 ± 1.5% for $CD4^+$ TT peptide-specific T cell line ZH-P1 (Fig. 6*C*) and 58 ± 6% for $CD8^+CD25^-$ MART1-peptide specific T cell clone ZH-7/2 (Fig. 6*C*). The mean percentages of inhibition of $CD4^+CD25^-$- and $CD8^+CD25^-$-specific T cells by syngeneic R0 or R1 T cells after stimulation with PHA peptide were similar to those obtained after peptide stimulation (data not shown).

To determine whether rapamycin induces suppressor T cells in $CD4^+CD25^-$ T cells, we analyzed inhibition of fresh allogeneic $CD4^+CD25^-$ T cell or $CD8^+CD25^-$ T cell proliferation in response to OKT3, anti-CD28 mAb, and IL-2 by $CD4^+CD25^-$ T cells after 7 days or 3 wk of expansion, respectively, in the presence or absence of rapamycin. CFSE-labeled responders stimulated with OKT and anti-CD28 Ab were cultured alone or coculture with $CD4^+CD25^-$ R0 or R1 cells at the ratio of 1:1 (Fig. 7). No evidence was obtained for induction of suppressor T cells in these cultures as indicated by low levels of suppressor activity in R1 cocultures (R0 cocultures not shown). The data indicated that rapamycin does not induce suppressor T cells in $CD4^+CD25^-$ populations during a 7-day or 3-wk incubation.

Next, we determined the suppressive activities of R1 T cells on cytokine secretion using IFN-γ ELISPOT assays. In contrast to R0 T cells, both syngeneic (Fig. 8) and allogeneic (data not shown) R1 T cells strongly inhibited IFN-γ secretion of $TT_{947-967}$ peptide-specific $CD4^+$ T cells (99.1 ± 0.5 and 97.3 ± 1%, respectively; Fig. 8, *top*) and Melan-A/$MART1_{26-35}$ peptide-specific $CD8^+$ T cells (98.7 ± 3 and 95.8 ± 0.6%, respectively; Fig. 8, *bottom*) upon peptide stimulation. Similar results were obtained upon stimulation with PHA (data not shown).

IL-10 expression by MACS-purified $CD4^+CD25^+$ T cells expanded by rapamycin

To distinguish nTreg cells from IL-10-secreting Treg cells (9), intracellular IL-10 expression was determined in both fresh $CD4^+CD25^+$ and in vitro-expanded R0 and R1 T cells after stimulation with LPS. Neither fresh $CD4^+CD25^+$ T cells nor R0 and R1 T cells after in vitro expansion expressed IL-10 as measured by flow cytometry (data not shown).

TCRVβ repertoire of MACS-purified $CD4^+CD25^+$ T cells expanded by rapamycin

TCRVβ chain repertoire of R0 and R1 T cells was determined using Vβ chain-specific Abs in a flow cytometry-based assay. We

found that R1 T cells after a 3-wk expansion displayed a polyclonal TCRVβ chain repertoire, comparable to $CD4^+CD25^+$ T cells of fresh PBMC and to 3 wk-expanded R0 T cells of the same donor (data not shown).

Discussion

We demonstrate here that human $CD4^+CD25^{high}Foxp3^+$ nTreg cells upon stimulation via TCR and CD28 or by IL-2 undergo rapid and robust expansion when rapamycin is added. Purified $CD4^+CD25^+$ T cells exposed to rapamycin: 1) exhibit a strong proliferative capacity; 2) show a $Foxp3^+GITR^+CTLA^-4^+$ phenotype; 3) have a potential for homing to lymph node (LN) as indicated by CD62L and CCR7 expression; 4) mediate potent suppressive activities against syngeneic or allogeneic $CD4^+$ and $CD8^+$ T cells, T cell clones or populations with defined antigenic specificity as well as polyclonal T cells with various antigenic specificities. In contrast, purified $CD4^+CD25^+$ T cells cultured in the absence of rapamycin (R0) are mainly composed of nonsuppressor T cells with a $CD25^{low}$ phenotype. The ex vivo use of rapamycin as outlined here should open the avenue for the in vitro generation of highly efficient nTreg cell populations in humans.

In the last few years, several strategies have been developed for long-term culture and polyclonal expansion of human nTreg cells (29–31). It has been demonstrated that the combined stimulation via TCR, CD28, and IL-2R is required to transiently induce human Treg proliferation and this strategy is especially effective with anti-CD3/CD28 beads or artificial APC (29–31). MACS-based purification has previously been used to obtain enriched $CD4^+CD25^+$ T cells for in vitro expansion. However, though straightforward, this method results in a considerable contamination with conventional $CD4^+CD25^{low}$ T cells. Consequently, the addition of high doses of IL-2 may promote an overgrowth of cells with modest or no suppressor activity. Thus, Battaglia et al. (25) showed that cultured murine $CD4^+CD25^+$ T cells not exposed to rapamycin lose their suppressive function. Others, using sorting for selection of only $CD4^+CD25^{high}$ cells, were able to generate suppressor cell lines from adult blood (32). However, as previously noted, even sorted populations may contain a mix of conventional and Treg cells (32), and relatively few $CD25^{high}$ T cells are recovered by this technique. In contrast, MACS separation of human $CD4^+CD25^+$ T cells followed by a large-scale expansion in the presence of rapamycin yields a population of cells with a defined phenotype and strong suppressive function.

Our data suggest that in the presence of rapamycin (1 nM), human Treg subsets present in the peripheral blood survive and expand, while the other T cells (i.e., $CD4^+CD25^{low}$) are inhibited from proliferation. We demonstrated that fresh or activated $CD4^+CD25^-$ T cells exposed to rapamycin (1 nM) in the presence of CD3/CD28 beads and 1000 IU/ml IL-2 do not proliferate, compared with $CD4^+CD25^-$ T cells cultured under the same conditions but in the absence of rapamycin. In contrast, rapamycin-exposed human $CD4^+CD25^{high}$ T cells vigorously expand for at least 3 wk in culture. Rapamycin also enhances PHA-stimulated expansion of Treg cells. We hypothesize that this expansion of $CD4^+CD25^{high}$ T cells results from rapamycin-driven selection of T cells at a unique state of activation, with rapamycin-induced apoptosis of $CD4^+CD25^-$ T cells. Sensitivity of these T cells to apoptosis, as indicated by ANX V binding, and resistance to apoptosis of fresh as well as rapamycin-expanded $CD4^+CD25^{high}$ T cells, suggest that the mechanism of rapamycin-based selection involves the death pathway. It is also noteworthy that rapamycin concentrations higher than 10 nM significantly decreased expansion rates of $CD4^+CD25^{high}$ T cells, while doses lower than 1 nM were not effective in inducing their expansion in our hands (data not shown). This indicates the existence of a threshold for rapamycin-mediated responses that could be related to the activation state of $CD4^+CD25^+$ T cells. Importantly, Treg cultured in the presence of IL-2 without rapamycin do not mediate suppression, while RT cells are highly suppressive (Fig. 6). In contrast to the findings reported by Valmori et al. (33), we demonstrate that the suppressive function of R1 T cells is maintained after rapamycin withdrawal (data not shown).

Phenotypic characteristics of RT cells are qualitatively similar to those of freshly isolated $CD4^+CD25^{high}$ T cells. We show here that rapamycin-expanded nTreg, like fresh nTreg, show high expression levels of CD25, Foxp3, CD62L, CD45RO, GITR, CTLA-4, CCR-7, Fas, and HLA-DR. In contrast, $CD4^+CD25^+$ T cells expanded in the absence of rapamycin express significantly lower levels of these markers. Furthermore, $CD4^+CD25^-$ and $CD4^+CD25^{low}$ T cells expanded in the presence of rapamycin showed low marker expression (Fig. 5*C*). This observation suggests that rapamycin promotes survival of a subset of $CD4^+CD25^{high}$ T cells that constitutively express high levels of the markers characterizing the suppressor phenotype. Furthermore, RT cells displayed a broad TCRVβ usage similar to that of $CD4^+CD25^-$ from fresh PBMC. As this TCRVβ chain repertoire remained virtually unchanged after in vitro expansion in the presence of rapamycin, it appears that RT cells represent a polyclonal expansion of T cells originating from precursors present in the peripheral blood.

RT cells expressed significantly higher levels of CD95 (Apo-1/Fas) molecules on the surface as compared with R0 T cells. Upon TCR stimulation of $CD4^+CD25^{high}$ cells or in RT cells, we observed no significant activation-induced cell death in agreement with recent findings (34), suggesting that these T cells were resistant to apoptosis despite high levels of CD95 expression. In contrast, rapamycin markedly accelerated apoptosis in the $CD4^+CD25^-$ T cell cultures, as shown by ANX V binding, while expanded RT cells remained alive. Similar apoptosis-mediated depletion of activated T cells and, consequently, an increase in $CD4^+CD25^+$ T cells upon rapamycin treatment has been described in allotransplant and autoimmune animal models (21, 35).

It is believed that successful expansion of Treg is a prerequisite for future adoptive transfers of these cells in human diseases. In transplantation or autoimmunity, expansion and transfers of Treg might be beneficial, while in cancer, their attenuation could allow for recovery of antitumor immune responses. After 1–3 wk of expansion, T cells exposed to rapamycin (RT cells) represented a pure population of $CD4^+CD25^{high}Foxp3^+$ T cells ($\geq$98%). Further phenotypic analysis showed that a considerable fraction expressed CD62L and CCR7, two receptors also present on T cells and some subsets of memory T cells and considered to be responsible for lymphocyte homing to secondary lymphoid organs (36). Indeed, accumulating data in animal models systems of autoimmunity or allotransplantation suggest that the LN is a critical site for tolerance induction (36, 37). The phenotype of human RT cells suggests that these cells could migrate to secondary lymphoid organs and to exert suppressive function in a site-specific manner.

Although our results indicate that rapamycin can be used for the expansion of Treg cells with a defined phenotype and suppressive functions from the peripheral blood of normal volunteers, in vitro expansion of Treg from blood of subjects with disease may not be equally feasible. In patients with autoimmune diseases, these cells might be less responsive or present in fewer numbers (38, 39). We are in the process of developing a protocol for a large-scale expansion of human Treg with rapamycin, and our preliminary data indicate that a 10^5 expansion index after 3 wk of culture is achievable with peripheral blood-derived $CD4^+CD25^+$ T cells of normal

donors. Also, it will be of interest to expand nTreg cell subsets isolated from cancer patients, especially patients with leukemia treated with hemopoietic stem cell transplantation. A possibility of therapy with expanded Treg to ameliorate graft-vs-host disease has been considered (40). In addition to therapeutic use, the availability of purified Treg in numbers sufficient for detailed phenotypic and functional analysis of these cells and understanding of their mode of action or Ag specificity is scientifically important.

In summary, we have shown that human polyclonal $CD4^+CD25^+$ nTreg cells capable of mediating potent suppression survive and readily proliferate upon exposure to rapamycin. Expanded $CD4^+CD25^{high}$ nTreg cells obtained from the peripheral circulation of normal donors show a "characteristic" suppressor phenotype and maintain expression of LN homing receptors. The data presented here may considerably accelerate the development of immunotherapeutic approaches for the treatment of autoimmune diseases or posttransplant alloreactions by the adoptive transfer of nTreg cells.

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Disclosures

The authors have no financial conflict of interest.

References

1. Shevach, E. M. 2002. $CD4^+CD25^+$ suppressor T cells: more questions than answers. *Nat. Rev. Immunol.* 2: 389–400.
2. von Herrath, M. G., and L. C. Harrison. 2003. Antigen-induced regulatory T cells in autoimmunity. *Nat. Rev. Immunol.* 3: 223–232.
3. Sakaguchi, S., N. Sakaguchi, J. Shimizu, S. Yamazaki, T. Sakihama, M. Itoh, Y. Kuniyasu, T. Nomura, M. Toda, and T. Takahashi. 2001. Immunologic tolerance maintained by $CD25^+CD4^+$ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol. Rev.* 182: 18–32.
4. Zou, W. 2006. Regulatory T cells, tumour immunity and immunotherapy. *Nat. Rev. Immunol.* 6: 295–307.
5. Woo, E. Y., C. S. Chu, T. J. Goletz, K. Schlienger, H. Yeh, G. Coukos, S. C. Rubin, L. R. Kaiser, and C. H. June. 2001. Regulatory $CD4^+CD25^+$ T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. *Cancer Res.* 61: 4766–4772.
6. Liyanage, U. K., T. T. Moore, H. G. Joo, Y. Tanaka, V. Herrmann, G. Doherty, J. A. Drebin, S. M. Strasberg, T. J. Eberlein, P. S. Goedegebuure, and D. C. Linehan. 2002. Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *J. Immunol.* 169: 2756–2761.
7. Schaefer, C., G. G. Kim, A. Albers, K. Hoermann, E. N. Myers, and T. L. Whiteside. 2005. Characteristics of $CD4^+CD25^+$ regulatory T cells in the peripheral circulation of patients with head and neck cancer. *Br. J. Cancer* 92: 913–920.
8. Curiel, T. J., G. Coukos, L. Zou, X. Alvarez, P. Cheng, P. Mottram, M. Evdemon-Hogan, J. R. Conejo-Garcia, L. Zhang, M. Burow, et al. 2004. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat. Med.* 10: 942–949.
9. Levings, M. K., R. Sangregorio, F. Galbiati, S. Squadrone, R. de Waal Malefyt, and M. G. Roncarolo. 2001. IFN-α and IL-10 induce the differentiation of human type 1 T regulatory cells. *J. Immunol.* 166: 5530–5539.
10. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J. E. de Vries, and M. G. Roncarolo. 1997. A $CD4^+$ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389: 737–742.
11. Jonuleit, H., E. Schmitt, M. Stassen, A. Tuettenberg, and J. Knop. 2001. Identification and functional characterization of human $CD4^+CD25^+$ T cells with regulatory properties isolated from peripheral blood. *J. Exp. Med.* 193: 1285–1294.
12. 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.
13. Levings, M. K., R. Sangregorio, and M. G. Roncarolo. 2001. Human $CD25^+CD4^+$ 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.
14. Chen, M. L., M. J. Pittet, L. Gorelik, R. A. Flavell, R. Weissleder, H. von Boehmer, and H. K. Khazaie. 2005. Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-β signals in vivo. *Proc. Natl. Acad. Sci. USA* 102: 419–424.
15. Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of $CD4^+CD25^+$ regulatory T cells. *Nat. Immunol.* 4: 330–336.
16. 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.
17. Wang, H. Y., D. A. Lee, G. Peng, Y. Li, Y. Kiniwa, E. M. Shevach, and R. F. Wang. 2004. Tumor-specific human $CD4^+$ regulatory T cells and their ligands: implications for immunotherapy. *Immunity* 20: 107–118.
18. Zhou, G., C. G. Drake, and H. I. Levitsky. 2006. Amplification of tumor-specific regulatory T cells following therapeutic cancer vaccines. *Blood* 107: 628–636.
19. Godfrey, W. R., D. J. Spoden, Y. G. Ge, S. R. Baker, B. Liu, B. L. Levine, C. H. June, B. R. Blazar, and S. B. Porter. 2005. Cord blood $CD4^+CD25^+$-derived T regulatory cell lines express FoxP3 protein and manifest potent suppressor function. *Blood* 105: 750–758.
20. Abraham, R. T., and G. J. Wiederrecht. 1996. Immunopharmacology of rapamycin. *Annu. Rev. Immunol.* 14: 483–510.
21. Zheng, X. X., A. Sanchez-Fueyo, M. Sho, C. Domenig, M. H. Sayegh, and T. B. Strom. 2003. Favorably tipping the balance between cytopathic and regulatory T cells to create transplantation tolerance. *Immunity* 19: 503–514.
22. Wood, K. J., and S. Sakaguchi. 2003. Regulatory T cells in transplantation tolerance. *Nat. Rev. Immunol.* 3: 199–210.
23. 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.
24. Battaglia, M., A. Stabilini, E. Draghici, B. Migliavacca, S. Gregori, E. Bonifacio, and M. G. Roncarolo. 2006. Rapamycin and interleukin-10 treatment induces T regulatory type 1 cells that mediate antigen-specific transplantation tolerance. *Diabetes* 55: 40–49.
25. Battaglia, M., A. Stabilini, and M. G. Roncarolo. 2005. Rapamycin selectively expands $CD4^+CD25^+FoxP3^+$ regulatory T cells. *Blood* 105: 4743–4748.
26. Altman, J. D., P. A. Moss, P. J. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael, and M. M. Davis. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274: 94–96.
27. Zippelius, A., P. Batard, V. Rubio-Godoy, G. Bioley, D. Lienard, F. Lejeune, D. Rimoldi, P. Guillaume, N. Meidenbauer, A. Mackensen, et al. 2004. Effector function of human tumor-specific CD8 T cells in melanoma lesions: a state of local functional tolerance. *Cancer Res.* 64: 2865–2873.
28. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401: 708–712.
29. 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.
30. 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: 895–903.
31. Earle, K. E., Q. Tang, X. Zhou, W. Liu, S. Zhu, M. L. Bonyhadi, and J. A. Bluestone. 2005. In vitro expanded human $CD4^+CD25^+$ regulatory T cells suppress effector T cell proliferation. *Clin. Immunol.* 115: 3–9.
32. 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.
33. 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.
34. Fritzsching, B., N. Oberle, N. Eberhardt, S. Quick, J. Haas, B. Wildemann, P. H. Krammer, and E. Suri-Payer. 2005. In contrast to effector T cells, $CD4^+CD25^+FoxP3^+$ regulatory T cells are highly susceptible to CD95 ligand- but not to TCR-mediated cell death. *J. Immunol.* 175: 32–36.
35. Rabinovitch, A., W. Suarez-Pinzon, A. M. Shapiro, R. V. Rajotte, and R. Power. 2002. Combination therapy with sirolimus and interleukin-2 prevents spontaneous and recurrent autoimmune diabetes in NOD mice. *Diabetes* 51: 638–645.
36. Ochando, J. C., A. C. Yopp, Y. Yang, A. Garin, Y. Li, P. Boros, J. Llodra, Y. Ding, S. A. Lira, N. R. Krieger, and J. S. Bromberg. 2005. Lymph node occupancy is required for the peripheral development of alloantigen-specific $FoxP3^+$ regulatory T cells. *J. Immunol.* 174: 6993–7005.
37. Jonuleit, H., E. Schmitt, G. Schuler, J. Knop, and A. H. Enk. 2000. Induction of interleukin 10-producing, nonproliferating $CD4^+$ T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J. Exp. Med.* 192: 1213–1222.
38. 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.
39. 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.
40. Steiner, D., N. Brunicki, B. R. Blazar, E. Bachar-Lustig, and Y. Reisner. 2006. Tolerance induction by third-party "off-the-shelf" $CD4^+CD25^+$ Treg cells. *Exp. Hematol.* 34: 66–71.