



HIT YOUR TARGET WITH CYTEK
PAY ONLY FOR WHAT YOU NEED

ONE-LASER, UP TO 9 COLOR
NL-1000 FLOW CYTOMETRY SYSTEM
FOR JUST \$49.5K

LEARN MORE



CD25⁻ T Cells Generate CD25⁺Foxp3⁺ Regulatory T Cells by Peripheral Expansion

Maria A. Curotto de Lafaille, Andreia C. Lino, Nino Kutchukhidze and Juan J. Lafaille

This information is current as of October 15, 2019.

J Immunol 2004; 173:7259-7268; ;
doi: 10.4049/jimmunol.173.12.7259
<http://www.jimmunol.org/content/173/12/7259>

References This article **cites 58 articles**, 31 of which you can access for free at:
<http://www.jimmunol.org/content/173/12/7259.full#ref-list-1>

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
<http://jimmunol.org/subscription>

Permissions Submit copyright permission requests at:
<http://www.aai.org/About/Publications/JI/copyright.html>

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
<http://jimmunol.org/alerts>

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2004 by The American Association of
Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.



CD25⁻ T Cells Generate CD25⁺Foxp3⁺ Regulatory T Cells by Peripheral Expansion¹

Maria A. Curotto de Lafaille,² Andreia C. Lino, Nino Kutchukhidze, and Juan J. Lafaille²

Naturally occurring CD4⁺ regulatory T cells are generally identified through their expression of CD25. However, in several experimental systems considerable T_{reg} activity has been observed in the CD4⁺CD25⁻ fraction. Upon adoptive transfer, the expression of CD25 in donor-derived cells is not stable, with CD4⁺CD25⁺ cells appearing in CD4⁺CD25⁻ T cell-injected animals and vice versa. We show in this study that CD25⁺ cells arising from donor CD25⁻ cells upon homeostatic proliferation in recipient mice express markers of freshly isolated T_{reg} cells, display an anergic state, and suppress the proliferation of other cells in vitro. The maintenance of CD25 expression by CD4⁺CD25⁺ cells depends on IL-2 secreted by cotransferred CD4⁺CD25⁻ or by Ag-stimulated T cells in peripheral lymphoid organs. *The Journal of Immunology*, 2004, 173: 7259–7268.

Regulatory T cells (T_{reg}),³ also called suppressor T cells, negatively modulate immune responses (1–4). The best-characterized, naturally occurring T_{reg} cells are CD4⁺ T cells of thymic origin that constitutively express CD25 and are unresponsive to stimulation in vitro (5–8). CD4⁺CD25⁺ T_{reg} cells constitutively express the transcription factor Foxp3 and the coreceptor CTLA-4 (9–13). Genetic deficiencies in these two genes cause profound immune dysregulation, leading to autoimmune diseases and allergy (14–18). T cells with T_{reg} activity are also contained in the CD4⁺CD25⁻ T cell population of normal animals. The regulatory T cell function of CD4⁺CD25⁻ T cells has been demonstrated in animal models of autoimmune encephalomyelitis (19, 20), diabetes (21–23), allergy (24), and inflammatory bowel disease (25). However, although CD4⁺CD25⁻ cells produced by depletion of CD25⁺ T cells can exert regulatory activity in several disease models, genetically deficient CD25^{-/-} cells are unable to do so (26). Thus, CD25 must be expressed at some point for T cells to exert suppression.

Upon transfer of purified CD4⁺CD25⁺ cells into T cell-deficient mice, scurfy mice, or TCR transgenic RAG^{-/-} mice, a substantial fraction of the cells become CD25⁻. Conversely, transfer of purified CD4⁺CD25⁻ cells into T cell-deficient mice generates a population of cells expressing CD25 (10, 25–28). CD25 expression appears to be more stable upon transfer of purified CD4⁺CD25⁺ T cells into wild-type mice (29). Laurie et al. (30) depleted CD4⁺CD25⁺ T cells by in vivo administration of anti-CD25 Abs to thymectomized BALB/c recipients and observed that CD25⁺ T cells returned to pretreatment proportion and function-

ality 48 days after Ab injection. In this and the previous cases it is unclear whether the CD25⁺ cells derived from the outgrowth of a small residual CD25⁺ population or the conversion of CD25⁻ into CD25⁺ T cells. The biological properties of the cells that acquired CD25 expression through homeostatic proliferation are not well known; likewise, the factors that influence the degree of CD25 marker switching remain poorly understood.

To study these issues we used an experimental system, the T/B monoclonal mice, in which we showed that both CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells display in vivo suppressive activity. On the BALB/c background, T/B monoclonal mice produce hyper-Th2 and IgE responses after a single immunization. These hyperresponses can be prevented by adoptive transfer of total CD4⁺, purified CD4⁺CD25⁺, or CD4⁺CD25⁻ T cells from BALB/c mice (24). T/B monoclonal mice harbor an I-A^d-restricted, monoclonal, OVA-specific T cell repertoire that lacks CD4⁺CD25⁺ T cells, and a monoclonal B cell repertoire specific for a linear peptide from influenza virus hemagglutinin (HA). T/B monoclonal mice were derived by crossing DO11.10 OVA-specific TCR transgenic mice, 17/9 HA-specific Ig H and L chain knockin mice, and RAG-1^{-/-} mice (24).

We show in this study that donor polyclonal CD4⁺CD25⁻ cells can convert in vivo to CD4⁺CD25⁺ upon homeostatic proliferation; the newly generated CD4⁺CD25⁺ cells are phenotypically and functionally equivalent to naturally occurring T_{reg} cells. The expression of CD25 in T_{reg} cells, which correlates with their T_{reg} ability, depends on IL-2 secreted by cotransferred CD25⁻ or by Ag-stimulated conventional T cells in peripheral lymphoid organs.

Materials and Methods

Mice

The T/B monoclonal mice (17/9 DO11.10 RAG-1^{-/-}) were previously described (24). T/B monoclonal mice harbor monoclonal populations of T and B lymphocytes that are specific for chicken OVA_{323–339} and HA of influenza virus, respectively. BALB/c Thy1.2 mice and BALB/c IL-2^{-/-} mice were purchased from The Jackson Laboratory (Bar Harbor, ME). BALB/c Thy1.1 mice were obtained from Dr. F. Zavala (New York University, New York, NY). TCRαβ-deficient mice were backcrossed to the BALB/c background in our facility. All mice were bred and housed at the Skirball specific pathogen-free animal facility.

Cell purification and adoptive transfer

BALB/c CD4⁺CD25⁺ and CD4⁺CD25⁻ lymphocytes were purified from spleen and lymph nodes by MACS using Miltenyi reagents and a Vario MACS apparatus (Miltenyi Biotec, Auburn, CA).

Molecular Pathogenesis Program, Skirball Institute, and Department of Pathology, New York University School of Medicine, New York, NY 10016

Received for publication July 16, 2004. Accepted for publication September 24, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by the National Institute of Allergy and Infectious Disease, National Institutes of Health, the National Multiple Sclerosis Society, and the Dana-Goldsmith Foundation (all to J.J.L.). A.C.L. is a recipient of Predoctoral Fellowship SFRH/BD/13129/2003 from Fundacao para a Ciencia e a Tecnologia, Portugal.

² Address correspondence and reprint requests to Dr. Maria A. Curotto de Lafaille or Dr. Juan J. Lafaille, Molecular Pathogenesis Program, Skirball Institute, and Department of Pathology, New York University School of Medicine, New York, NY 10016. E-mail address: curotto@saturn.med.nyu.edu or lafaille@saturn.med.nyu.edu

³ Abbreviations used in this paper: T_{reg}, regulatory T cell; HA, hemagglutinin from influenza virus; MFI, mean fluorescence intensity; GITR, glucocorticoid-induced TNFR.

To purify spleen and lymph node CD4⁺CD25⁻ cells by MACS, the cells were first incubated with FITC-labeled Abs to CD8, B220, and CD25 (in some experiments Abs to CD11b, CD11c, and TER119 were also added), followed by incubation with anti-FITC magnetic beads. The cells were then passed through the first depletion column, incubated with more anti-FITC magnetic beads, and passed through a second depletion column. Thymic CD4⁺CD25⁻ cells were purified by depletion of CD8⁺ and CD25⁺ cells using a similar two-step protocol. The unbound fraction typically contained 85–95% CD4⁺CD25⁻ cells, 0.1–0.3% CD25^{low} cells, and no CD25^{high} cells. To purify CD4⁺CD25⁺ cells, spleen and lymph node cell suspensions were first depleted of CD8⁺ and B220⁺ cells by MACS, incubated with PE-anti-CD25 Abs followed by anti-PE magnetic beads, and subsequently purified as the bound fraction from a MACS column. The purity of the CD25⁺ population was >95%.

CD25⁺, CD25⁻CD45RB^{high}, and CD25⁻CD45RB^{low} fractions from BALB/c mice used in the experiments shown in Fig. 5 were purified by cell sorting in a MoFlo cytometer (DakoCytomation, Carpinteria, CA) at New York University School of Medicine with ≥99% purity. Typically, 1–5 × 10⁶ purified cells were injected i.v. into recipient mice. All recipient mice were treated with broad-spectrum antibiotic (Bactrim; Sulfatrim Pediatric Suspension; Alpharma USPD, Baltimore, MD). Immunization of T/B monoclonal mice was performed the day after cell transfer, i.p. with 100 μg of cross-linked OVA-HA in alum (24).

CFSE labeling

Cells purified as described above were labeled with CFSE (Molecular Probes, Eugene, OR) by incubating 10⁷ cells/ml in PBS with 5 μM CFSE resuspended in DMSO. Labeling was stopped with medium containing 10% FCS.

Immunostaining and RNA analysis

Spleen and lymph node cells from recipient mice were analyzed by flow cytometry using fluorochrome-labeled Abs purchased from BD Pharmingen (San Diego, CA), Caltag Laboratories (Palo Alto, CA), and R&D Systems (Minneapolis, MN). The samples were analyzed using a FACSCalibur or an LSR II cytometer (BD Biosciences, Mountain View, CA). For quantitative mRNA expression analysis, donor-derived CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were MoFlo-sorted. RNA was extracted from the samples using TRIzol, and cDNA was synthesized using standard procedures. The expression of Foxp3, CTLA-4, glucocorticoid-induced TNFR (GITR), and β-actin was determined by quantitative real-time PCR using the primers below.

Expression was normalized to the level of β-actin in each sample: Foxp3, ACTGGGGTCTCTCCCTCAA, CGTGGGAAGGTGCAGAGTAG; CTLA-4, GTTGGGGGCATTTTCACATA, TTTTACAGTTTCCTGGTCTC; and β-actin, TGACAGGATGCAGAAGGAGA, GACTTGCCTCAGGAGGAG.

In vitro suppression assays

Donor-derived CD4⁺CD25⁺ and CD4⁺CD25⁻ cells from spleen and lymph nodes of recipient mice were MoFlo-sorted. Proliferation assays were set up in 96-well, round-bottom plates and contained, per well, 1 × 10⁴ responder cells, 2 × 10⁴ APC (mitomycin C-treated spleen cells from TCRαβ-deficient BALB/c mice), and anti-CD3 Ab at a concentration of 0.5 μg/ml. Putative suppressor cells were cocultured at responder:suppressor ratios of 1:1, 1:0.3, and 1:0.1. Proliferation was determined by adding [³H]thymidine on the third day of culture and determining incorporation 6 h later.

Results

Peripheral CD4⁺CD25⁻ T cells generate CD4⁺CD25⁺ cells by peripheral expansion

We and others have observed that in conditions of homeostatic proliferation, CD25 expression is not stably maintained. Expanding CD4⁺CD25⁻ cells generate a population of CD25⁺ cells, although expanding CD25⁺ cells partially lose CD25 expression (25–28). To analyze the phenotypic and functional characteristics of the CD25⁺ cells derived from CD25⁻ cells, we adoptively transferred purified CD25⁻ T cells into host mice in which donor cells could undergo homeostatic expansion, such as RAG-1 knockout mice and T/B monoclonal mice. T/B monoclonal mice are DO11.10 TCR transgenic mice crossed with 17/9 Ig H and L chain knockin mice and RAG-1 knockout mice on the BALB/c genetic

background. T/B monoclonal mice are devoid of T_{reg} cells, and their peripheral T cells have a naive phenotype (24).

To investigate the relationship between homeostatic proliferation and peripheral conversion of CD25⁻ T cells to CD25⁺, we purified CD4⁺CD25⁻ cells from BALB/c mice (Fig. 1A), labeled

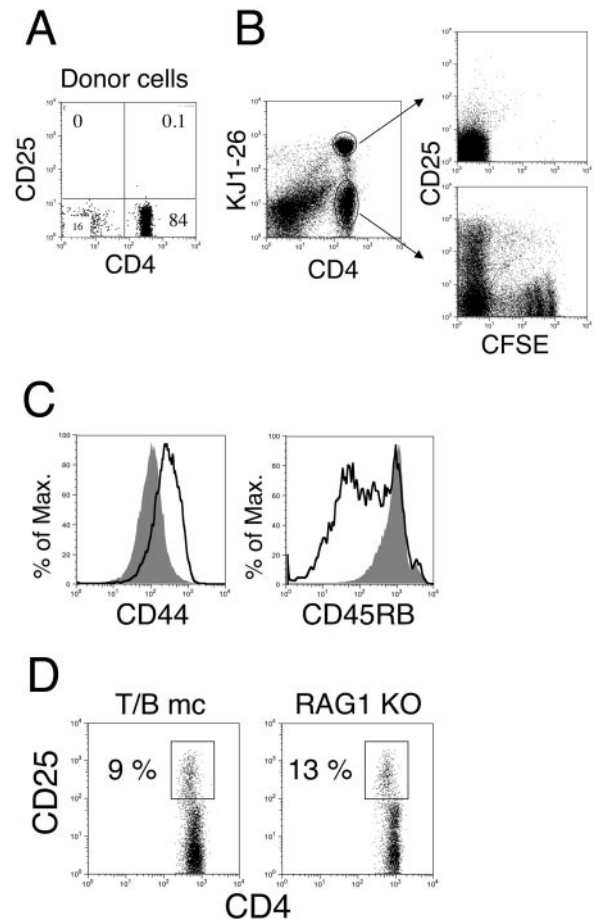


FIGURE 1. Conversion of CD4⁺CD25⁻ cells to CD25⁺ cells in T/B monoclonal and RAG-1^{-/-} recipient mice during peripheral expansion. *A*, CD4⁺CD25⁻ cells were purified from spleen and lymph nodes of BALB/c mice with ≤0.1% contamination by CD25⁺ cells. The cells were labeled with CFSE and transferred to T/B monoclonal mice (5 × 10⁶ cells/mouse; *n* = 3). One month later, recipient mice were killed, and spleen and lymph node cells were analyzed by flow cytometry. Representative analyses are shown in *B* and *C*. *B*, Cells were stained with anti-CD4, anti-CD25, and KJ1-26 Abs. The *left* plot shows the gates used for the analysis of endogenous cells (CD4⁺KJ1-26⁺) and the donor-derived cells (CD4⁺KJ1-26⁻). The *lower right* plot shows CFSE dilution and CD25 expression in the donor-derived population. The *upper right* plot shows endogenous cells (CFSE-negative and naive). *C*, Cells were stained with anti-CD4, anti-CD45RB, anti-CD44, and KJ1-26 Abs. Donor and endogenous populations were gated as described in *B*. The figure shows overlapping histograms of CD44 (*left*) and CD45RB (*right*) expression in donor cells (CD4⁺KJ1-26⁻; line) and endogenous cells (CD4⁺KJ1-26⁺; solid gray). *D*, CD4⁺CD25⁻ cells were purified from spleen and lymph nodes of BALB/c (Thy 1.1/1.2) mice and transferred to T/B monoclonal mice (T/B mc; Thy 1.2) and RAG-1^{-/-} mice (2 × 10⁶ cells/mouse; *n* = 3). Two weeks after immunization, the expression of CD25 in donor-derived CD4⁺ cells (Thy1.1⁺) was analyzed in peripheral blood samples stained with Abs to Thy1.1, CD4, and CD25. The plots show CD25 expression in donor-derived cells gated as CD4⁺Thy1.1⁺. Homeostatic proliferation of polyclonal CD4⁺ T cells in T/B monoclonal mice, and conversion of CD25⁻ into CD25⁺ cells in T/B monoclonal and RAG-1^{-/-} mice has been extensively confirmed in numerous experiments using both MACS- or MoFlo-sorted donor cells obtained from peripheral lymphoid organs or thymus.

them with CFSE, and transferred them to T/B monoclonal mice. Homeostatic proliferation of polyclonal CD4⁺ T cells in T/B monoclonal mice was demonstrated by CFSE dilution in the donor-derived population (gated as KJ1-26⁺CD4⁺ in Fig. 1B). Cells that became CD25⁺ were found in the population that underwent extensive proliferation (Fig. 1B). As in T cell-deficient hosts, cells that expanded in T/B monoclonal mice acquired a memory phenotype, expressing high levels of CD44 and low levels of CD45RB (Fig. 1C). In contrast, the endogenous OVA-specific KJ1-26⁺CD4⁺ cells maintained a naive phenotype (CD25⁻, CD44^{low}, CD45RB^{high}). Simultaneous transfer of purified CD4⁺CD25⁻ BALB/c cells into T/B monoclonal mice and RAG1^{-/-} mice demonstrated similar rates of conversion to CD25⁺ cells in both host strains (Fig. 1D).

It has been known for some time that the level of CD4 on the surface of CD4⁺CD25⁺ T_{reg} cells is lower than the level of CD4 on naive CD4⁺ T cells (for instance, see BALB/c cells in Fig. 2A). Upon injection of CD4⁺CD25⁻ cells obtained from secondary lymphoid organs (designated peripheral in Fig. 2A) or CD4⁺CD25⁻ single-positive cells from thymus (designated SP in Fig. 2A), we monitored the kinetics of appearance of CD4⁺CD25⁺ cells as well as the levels of CD4 surface expression. FACS analysis was performed on peripheral blood 5, 8, and 11 days after transfer of CD25⁻ T cells (Fig. 2A). Although the donor-derived population contained CD25⁺ cells on day 5, only on day 8 was the population expressing high CD25 and low CD4 evident. The kinetics of generation of CD25⁺ cells as well as the down-modulation of CD4 were similar regardless of whether CD25⁻ cells were obtained from peripheral lymphoid organs or thymus.

To demonstrate that the peripherally generated CD4⁺CD25⁺ cells derive from acquisition of CD25 expression by CD4⁺CD25⁻ T cells, rather than by expansion of contaminant CD25⁺ cells (usually 0.1–0.3% in the donor CD25⁻ population), we spiked purified BALB/c CD4⁺CD25⁻ Thy1.2 cells with 0.5% contaminant CD4⁺CD25⁺ Thy1.1 cells and transferred the mixed population to T/B monoclonal mice. After 10 days, contaminant CD4⁺Thy1.1⁺ cells constituted ~1% of total donor-derived cells (2-fold increase from initial input), whereas Thy1.2⁺ donor-derived cells contained >7% CD25⁺ cells (49-fold increase from initial input; Fig. 2B). Thus, CD25⁺ cells derived from donor CD25⁻ cells did not originate from preferential expansion of a few initial CD25⁺ cells. Instead, a fraction of CD25⁻ cells converted to CD25⁺ cells in the periphery of recipient mice. FACS analysis of TCR V α and V β gene usage in peripherally converted CD25⁺ cells showed a broad diversity, similar to the freshly isolated CD4⁺ cells from BALB/c mice, arguing against the conversion-expansion of a few clones of CD25⁻ donor cells (data not shown).

CD4⁺CD25⁺ T cells derived from peripherally expanded CD25⁻ cells are anergic and suppressive

We subsequently investigated whether the CD4⁺CD25⁺ cells arising from donor CD4⁺CD25⁻ cells displayed phenotypic and functional properties ascribed to freshly isolated CD4⁺CD25⁺ T_{reg} cells (9–13, 31–34). Analysis of RNA expression by real-time quantitative PCR in purified donor-derived cells demonstrated high levels of Foxp3 in the donor-derived CD25⁺, but not in the donor-derived CD25⁻ fraction (Fig. 3A). It is important to note that unlike other T_{reg} markers, Foxp3 is not expressed by activated conventional mouse CD4⁺ cells (9–11). Flow cytometric analysis showed that donor-derived CD25⁺ cells, but not CD25⁻ cells, expressed GITR, CD103, and CTLA-4 (Fig. 3B). Thus, phenotypically, CD4⁺CD25⁺ cells derived from homeostatic expansion of CD4⁺CD25⁻ cells are very similar to freshly isolated CD4⁺CD25⁺ T cells.

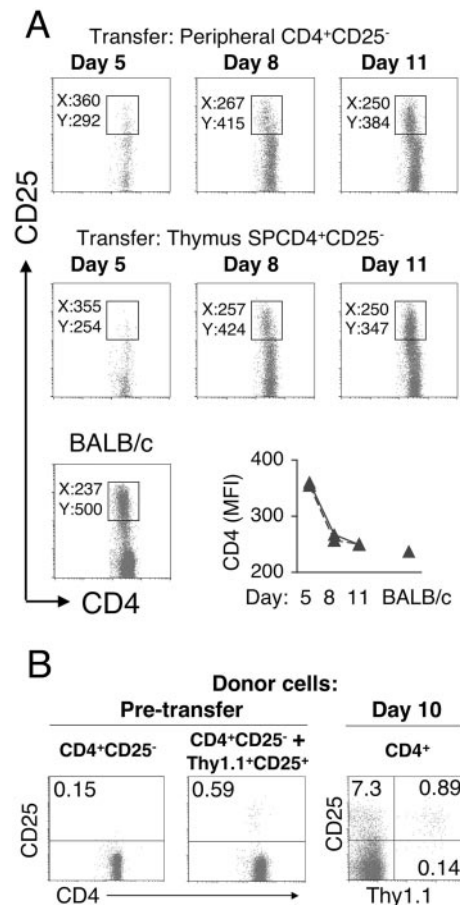


FIGURE 2. A, Kinetics of conversion of CD25⁻ cells into CD25^{high}CD4^{low} cells. Purified CD4⁺CD25⁻ cells from spleen and lymph nodes (peripheral) or from thymus of BALB/c mice were transferred to T/B monoclonal mice (2×10^6 cells/mouse). The expression of CD25 in donor-derived CD4⁺ cells was analyzed in peripheral blood samples on days 5, 8, and 11 after transfer. MFI values for CD25 (Y) and CD4 (X) in the CD25⁺ gate are indicated. The graphic on the bottom right shows the overlay of CD4 MFI (CD25⁺ cell gated) in donor-derived T cells from periphery (solid line) and thymus (dashed line). Note the overlapping curves, showing, in both cases, the decrease in CD4 MFI over time. The kinetic analysis is from a representative mouse from each group of four mice. Expression of CD4 (CD25⁺ cell gated) in peripheral blood of a BALB/c mouse is shown for comparison. B, Conversion, rather than selective expansion, of contaminant cells explains the appearance of CD25⁺ cells from a population of CD25⁻ donor cells. CD4⁺CD25⁻ cells were purified from spleen of BALB/c Thy1.2 mice. CD4⁺CD25⁺ cells were purified from spleen of BALB/c Thy1.1 mice. The purified cells were mixed in a ratio of 200:1 (CD25⁻:CD25⁺) and injected into T/B monoclonal mice. A total of 2×10^6 CD4⁺CD25⁻ cells was injected per mouse. Pretransfer donor cells (CD4⁺CD25⁻) before and after addition of the contaminant CD25⁺ Thy1.1⁺ cells are shown in the left and middle plots. The right plot shows CD25 expression in CD4⁺ Thy1.2⁺ and Thy1.1⁺ donor-derived CD4⁺ cells from spleen of recipient mice (day 10 after transfer; endogenous cells were excluded by staining with the anti-OVA-TCR Ab KJ1-26). The data are representative of two experiments.

The suppressive ability of CD25⁺ cells derived from CD25⁻ donor cells was tested in *in vitro* proliferation assays. In these assays, donor-derived CD4⁺CD25⁺ cells were as anergic and suppressive as freshly isolated BALB/c CD4⁺CD25⁺ cells (Fig. 3C). Little or no suppressor activity was detected in the donor-derived CD25⁻ population.

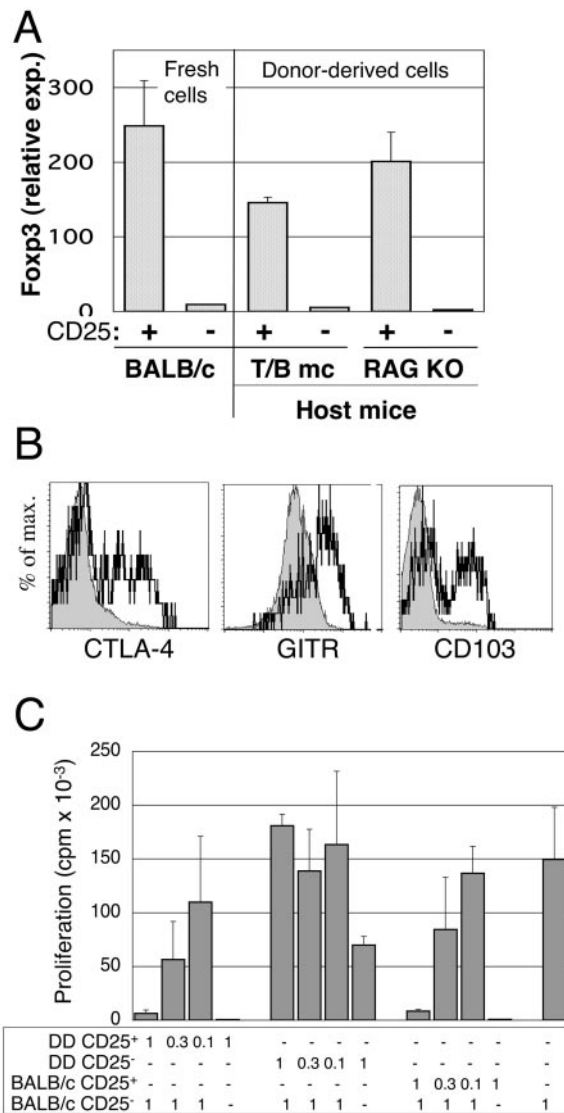


FIGURE 3. CD4⁺CD25⁺ cells derived from CD4⁺CD25⁻ cells have phenotypic and functional characteristics of T_{reg} cells. **A**, CD25⁺ cells derived from CD25⁻ cells express Fopx3 mRNA. CD4⁺CD25⁻ cells were purified from spleen and lymph nodes of BALB/c mice and transferred into T/B monoclonal mice and RAG-1^{-/-} mice. Donor-derived CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were isolated from T/B monoclonal mice or RAG-1^{-/-} mice 11 days after transfer. The expression of Fopx3 in mRNA from donor-derived cell samples as well as from freshly isolated CD4⁺CD25⁺ and CD4⁺CD25⁻ BALB/c cells was determined by real-time PCR. β -Actin amplification was used for normalization. The figure shows the average and SD of triplicate wells. **B**, CD25⁺ cells derived from CD25⁻ cells express GITR, CD103, and CTLA-4. CD4⁺CD25⁻ cells were purified from spleens of BALB/c mice and transferred into T/B monoclonal mice. Two weeks after transfer, the mice were killed, spleen cells were harvested, and the expression of T_{reg} markers (CTLA-4, CD103, and GITR) in the donor-derived population (CD4⁺KJ1-26⁻) was determined by flow cytometry. The figure shows histograms of gated CD4⁺KJ1-26⁻CD25⁺ (line) and CD4⁺KJ1-26⁻CD25⁻ (■) donor-derived cells. **C**, CD25⁺ cells derived from CD25⁻ cells are anergic and suppressive. Donor-derived CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were isolated from T/B monoclonal mice that had been transferred with CD4⁺CD25⁻ cells from BALB/c mice as in **A**. Donor-derived CD4⁺CD25⁺ and CD4⁺CD25⁻ cells as well as freshly isolated CD4⁺CD25⁺ BALB/c cells were cultured alone or were cocultured with 1×10^4 freshly isolated CD4⁺CD25⁻ BALB/c cells (responder cells) in wells containing anti-CD3 Abs and APC. Ratios of responder to suppressor cells were 1:1, 1:0.3, and 1:0.1. On the third day of culture, proliferation was measured by [³H]thymidine incorporation. Results are the average and SD of triplicate wells. DD, donor-derived.

Peripheral conversion of CD4⁺CD25⁻ T cells to CD4⁺CD25⁺ T cells in wild-type newborn mice

As shown above, peripheral conversion of polyclonal CD4⁺CD25⁻ to functional CD25⁺ T_{reg} cells occurs efficiently in mice with no T cells (such as RAG-deficient mice) or mice harboring large numbers of monoclonal T cells (such as T/B monoclonal mice). We investigated whether peripheral generation of T_{reg} cells from CD25⁻ T cells could take place in mice with normal immune systems.

To study the expansion and conversion of CD25⁻ cells in wild-type mice, CD4⁺CD25⁻ cells from BALB/c Thy1.1/1.2 mice were purified, labeled with CFSE (to follow cell division), and transferred into newborn and adult BALB/c Thy1.2 recipient mice.

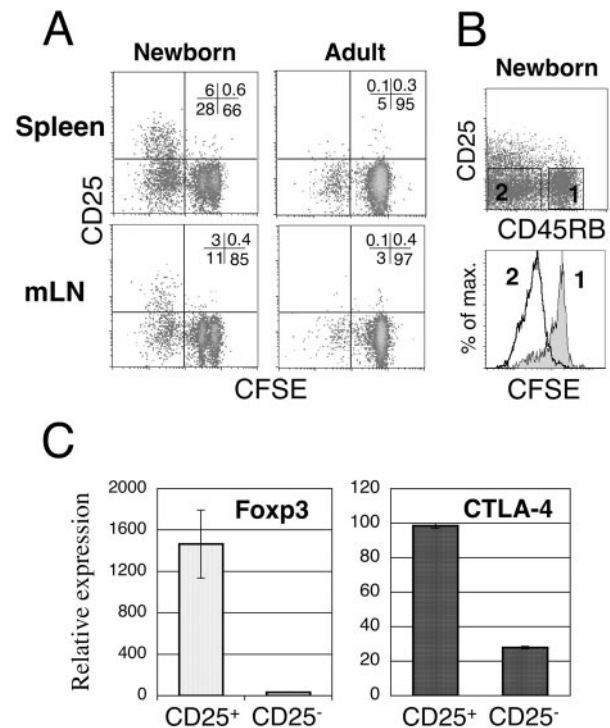


FIGURE 4. CD4⁺CD25⁻ cells transferred into wild-type BALB/c neonates give rise to CD4⁺CD25⁺CD45RB^{low} cells. CD4⁺CD25⁻ cells were purified from spleen and lymph nodes of BALB/c Thy1.1/1.2 mice, labeled with CFSE, and transferred into neonates ($0.5\text{--}1 \times 10^6$ /mouse) or adult (5×10^6 cells/mouse) BALB/c Thy1.2 mice. Three weeks later, mice were killed, and spleen and lymph node cells were analyzed by flow cytometry using Abs to CD4, CD25, CD45RB, and Thy1.1. **A**, Representative plots showing CFSE intensity and CD25 expression in gated donor cells (CD4⁺Thy1.1⁺) from spleens and lymph nodes of a neonatally injected mouse and an adult recipient mouse. **B**, Expression of CD45RB and CD25 in gated donor cells from a neonatally injected mouse is shown on the *top*. Cell divisions of fractions 1 and 2 are shown by histograms of CFSE dilution. CFSE dilution in gated donor-derived CD25⁻CD45RB^{high} (fraction 1) and CD25⁻CD45RB^{low} (fraction 2) cells from the same sample are shown on the *right panel*. CFSE dilution of CD25⁺CD45RB^{low} cells is shown in **A**, *left panels*, and is completely overlapping with the fraction 2 shown in **B**. Data are representative of three experiments. **C**, CD4⁺CD25⁻ cells transferred to BALB/c neonates give rise to CD25⁺Fopx3⁺ cells. CD4⁺CD25⁻ cells were purified from spleen and lymph nodes of BALB/c Thy1.1/1.2 mice and transferred into neonatal BALB/c Thy1.2 mice. One month after transfer, donor-derived Thy1.1⁺CD4⁺CD25⁺ and donor-derived Thy1.1⁺CD4⁺CD25⁻ cells were purified from spleen and lymph nodes. The levels of mRNA for Fopx3 and CTLA-4 in the samples were determined by real-time PCR as described. Expression levels (average \pm SD) relative to β -actin are shown.

In neonatally injected mice, the donor CD25⁻ population efficiently generated CD25⁺ cells within the proliferating (CFSE^{low}) cells (Fig. 4A). Very low proliferation and conversion occurred in adult mice. Cells that converted to CD25⁺ in neonatally injected mice were found in the CD45RB^{low} fraction of donor-derived CD4⁺ cells, similarly to naturally occurring T_{reg} cells (Fig. 4B). All CD45RB^{low} cells within the donor population had undergone three or more cell divisions at the time of analysis. Similarly to naturally occurring T_{reg} cells, CD25⁺ T cells derived from CD25⁻ T cells in normal newborn mice expressed high levels of Foxp3 and CTLA-4 (Fig. 4C). Thus, peripheral conversion of CD25⁻ into CD25⁺ T_{reg} cells occurs in wild-type mice in conditions of peripheral expansion, such as the neonatal period (35).

Naive T cells give raise to T_{reg} cells in vivo with delayed kinetics

Peripheral CD4⁺CD25⁻ T cells represent a heterogeneous population containing naive and memory cells. Among other markers, this heterogeneity can be visualized by the differential expression of CD45RB. In clean animal facilities, a large fraction of CD4⁺CD25⁻ T cells are naive T cells that express high levels of CD45RB (CD45RB^{high}), and the remaining CD4⁺CD25⁻ peripheral cells express intermediate or low levels of CD45RB (CD45RB^{low}). Peripheral CD4⁺CD25⁺ cells are CD45RB^{low} (12, 25). We sought to determine whether the CD45RB^{high} and CD45RB^{low} fractions of CD4⁺CD25⁻ cells had a different capacity to convert to CD25⁺ T_{reg} cells during homeostatic expansion. CD4⁺CD25⁻CD45RB^{high} and CD45RB^{low} cells from spleen and lymph nodes of BALB/c mice were purified by cell sorting and transferred into T/B monoclonal mice. Although a comparable fraction of CD45RB^{high}- and RB^{low}-derived CD4⁺ T cells expressed CD25 on day 6 after transfer, only CD25⁺ cells derived from CD25⁻CD45RB^{low} T cells had a phenotype similar to that of wild-type T_{reg} cells, such as higher CD25 and lower CD4 expression (Fig. 5A). However, 21 days after transfer, CD25⁺ cells derived from CD25⁻RB^{high} or RB^{low} cells had similar FACS profiles (Fig. 5A). Similarly, CD4⁺CD25⁺ T cells derived from donor CD25⁻CD45RB^{high} cells took a longer time to reach comparable expression levels of T_{reg} genes, Foxp3 and CTLA-4, than the cells derived from the CD25⁻CD45RB^{low} donor population (Fig. 5B). Similar results were obtained when CD4⁺CD25⁻CD45RB^{high} and CD45RB^{low} BALB/c cells were transferred into RAG1^{-/-} recipients (data not shown). We concluded that both CD45RB^{high} and CD45RB^{low} fractions of CD25⁻ cells are able to generate peripheral CD25⁺ cells with phenotype of T_{reg} cells, but the CD45RB^{low} fraction is (at least kinetically) more efficient.

CD25 expression in peripheral lymphoid organs is regulated by IL-2-producing effector-type T cells

It has been reported that upon transfer of CD25⁺ T cells into alymphoid recipients, the expression of CD25 is lost in a large part of the population unless CD4⁺CD25⁻CD45RB^{high} T cells are co-transferred (25). We observed similar down-regulation of CD25 expression when CD25⁺ cells were transferred into T/B monoclonal mice. Given the fact that the T/B monoclonal mice contain a large number of CD4⁺CD25⁻CD45RB^{high} T cells and virtually no CD4⁺CD25⁺ T cells, our finding was unexpected. The difference between the experiments was that the CD4⁺CD25⁻CD45RB^{high} cells in the T/B monoclonal mice were OVA-specific resting cells, whereas polyclonal T cells injected into alymphoid mice were undergoing homeostatic proliferation. Peripheral expansion of the polyclonal T cells would lead to, among other things, IL-2 production. To test whether IL-2 production by CD25⁻ T cells was necessary for the maintenance of CD25 expression by

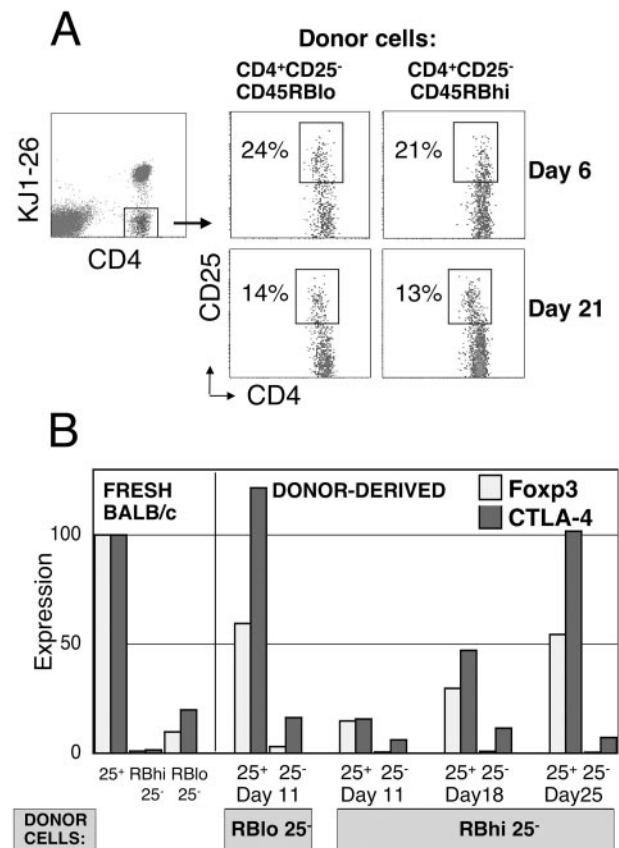


FIGURE 5. CD4⁺CD25⁻CD45RB^{high} naive T cells give raise to T_{reg} cells in vivo with delayed kinetics. **A**, CD4⁺CD25⁻CD45RB^{low} (RBlo) and CD4⁺CD25⁻CD45RB^{high} (RBhi) T cells were purified from BALB/c mice and transferred to T/B monoclonal mice (1 × 10⁶ cells/mouse). The expression of CD25 in donor-derived cells (CD4⁺ KJ1-26⁻) was monitored in peripheral blood of recipient mice. The figure shows representative FACS analysis of samples obtained 6 and 21 days after transfer. **B**, Cells were transferred as indicated in **A**. Donor-derived CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were sorted from spleens of recipient mice 11, 18, and 25 days after transfer. The expression of mRNA for Foxp3 and CTLA-4 in the samples was determined by real-time PCR. Purified CD4⁺CD25⁺, CD4⁺CD25⁻CD45RB^{low}, and CD4⁺CD25⁻CD45RB^{high} cells from spleen and LN cells of BALB/c mice were included in the analysis. Levels of Foxp3 and CTLA-4 are expressed as a percentage of the value obtained for fresh CD4⁺CD25⁺ cells from BALB/c mice. Data are representative of three experiments. Similar results were obtained in two experiments in which RAG-1^{-/-} mice were used as recipients.

CD4⁺CD25⁺ cells, we cotransferred a polyclonal population of CD25⁻ T cells from IL-2^{-/-} BALB/c Thy1.2 mice with purified IL-2⁺ CD4⁺CD25⁺ T cells from BALB/c Thy1.1 mice into T/B monoclonal mice. Although cotransfer of polyclonal CD25⁻ IL-2⁺ T cells allowed the maintenance of CD25 expression on 80% of CD25⁺ cells, IL-2^{-/-} T cells were unable to affect the frequency of CD25⁺ T cells among Thy1.1 cells (Fig. 6A). FACS analysis of the Thy1.1 donor population in spleens of recipient mice revealed that the cotransfer of CD25⁻ IL-2⁺ cells helped sustain Thy1.1 donor cells expressing high levels of CD25 and CTLA-4 (Fig. 6B). We conclude that polyclonal CD25⁻ cells help to maintain peripheral T_{reg} cells through IL-2 production.

Activation of naive T cells in vivo results in IL-2 production a few hours after stimulation (36). Thus, we hypothesized that immune responses may also affect T_{reg} cell homeostasis. As reported in other experimental systems, in T/B monoclonal mice, IL-2 production, and CD69 and CD25 expression are induced in

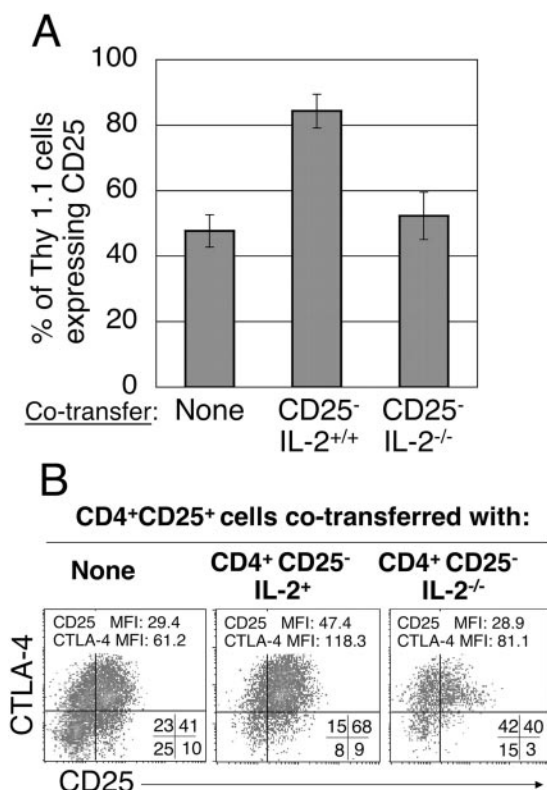


FIGURE 6. Wild-type CD4⁺CD25⁻ cells, but not IL-2-deficient CD4⁺CD25⁻ cells, help maintain a functional population of peripheral CD4⁺CD25⁺ T_{reg} cells. **A**, CD4⁺CD25⁺ cells from BALB/c Thy1.1 mice were transferred alone (0.5×10^6), or cotransferred with 2×10^6 CD4⁺CD25⁻ cells from IL-2^{+/+} or IL-2^{-/-} BALB/c Thy 1.2 mice into T/B monoclonal recipients. Ten days after transfer, the expression of CD25 in the Thy1.1⁺ donor population was determined by FACS analysis of peripheral blood samples. The figure shows the average and SD of the percentage of CD25⁺ cells within gated CD4⁺Thy1.1⁺ donor cells ($n = 3$). **B**, Mice from the groups described in **A** were killed 2 wk after transfer. The expression of CD25 and CTLA-4 in the CD4⁺Thy1.1⁺ donor population (originally CD25⁺) was analyzed in spleen samples of the recipient mice. Representative dot plots of gated CD4⁺Thy1.1⁺ cells are shown. A high percentage of Thy1.1⁺ cells expressing CD25 and CTLA-4 (upper right quadrant) were found in mice cotransferred with Thy1.2⁺ CD25⁻ IL-2^{+/+} cells, but not in mice cotransferred with Thy1.2⁺ CD25⁻ IL-2^{-/-} cells or in mice that received Thy1.1⁺ CD25⁺ cells alone.

OVA-specific T cells a few hours after immunization (24) (Fig. 7). Kinetic analysis of IL-2 mRNA expression after immunization of T/B monoclonal mice that had or had not been transferred with BALB/c splenocytes showed that IL-2 production by OVA-specific host cells peaked during the first day of immunization and decreased thereafter in the transferred and untransferred groups (Fig. 7A). In contrast, IL-4 production, a later differentiation event, was suppressed in the transferred group (24). The production of IL-2 protein by permeabilized OVA-specific T cells 14 h after immunization was verified by intracellular staining (Fig. 7B). The analysis of CD25 expression in the donor-derived polyclonal CD4⁺ cells from the transferred group demonstrates a higher CD25 expression per cell (as determined by the mean fluorescence intensity (MFI)) and a higher percentage of CD25⁺ cells (Fig. 7B, MFI of nonimmunized, 228; MFI of immunized samples, 502 and 656, respectively, for the samples represented with thinner and thicker lines).

Although most IL-2 production occurs early after immunization, the effects of immunization on the donor-derived CD4⁺ population

could be detected >1 wk later. Purified BALB/c CD4⁺CD25⁺, CD4⁺CD25⁻, or total CD4⁺ cells were transferred into T/B monoclonal mice. Half of each group was subsequently immunized with OVA-HA. The expression of CD25 in the donor-derived population was determined 9 days later by FACS analysis of peripheral blood samples. Immunization of T/B monoclonal mice with OVA-HA resulted in a higher frequency of donor-derived cells expressing CD25 (Fig. 7, B and C). The greatest effect was observed in mice transferred with purified CD25⁺ cells. Thus, immune responses of conventional CD4 cells could help sustain the pool of T_{reg} cells.

The suppressor function of CD25⁺ cells is regulated by IL-2-producing effector-type T cells

Having described the properties of CD4⁺CD25⁺ T cells that derive from CD4⁺CD25⁻ T cells upon homeostatic proliferation, we determined the properties of CD4⁺CD25⁻ T cells that originate upon transfer of CD4⁺CD25⁺ T cells. Specifically, we studied whether CD25⁻ cells derived from donor CD25⁺ cells that expanded in T/B monoclonal mice maintained the regulatory activity of their CD25⁺ predecessors. In addition, we determined whether immunization of the recipient T/B monoclonal mice with OVA-HA affected the suppressor activity of donor CD25⁺ cells. To address these issues we purified splenic CD4⁺CD25⁺ cells from BALB/c mice and transferred them into T/B monoclonal recipient mice. Half the recipient mice were immunized twice with OVA-HA (indicated I in Fig. 8), whereas the other half was not immunized (indicated NI in Fig. 8). Three days after the second immunization, donor-derived CD4⁺CD25⁻ and CD4⁺CD25⁺ cells were isolated from the recipient mice and tested in vitro for the expression of T_{reg}-associated genes, as well as for in vitro suppressor activity. Both donor-derived CD25⁺ and CD25⁻ cells expressed CTLA-4, CD103, and GITR, but expression levels were higher in the CD25⁺ population (Fig. 8A). The donor-derived CD25⁺ and CD25⁻ populations were tested in vitro for their ability to respond to anti-CD3 stimulation and to suppress the proliferation of freshly isolated CD25⁻ cells (from DO11.10 RAG^{-/-} mice). Donor-derived CD25⁺ cells from immunized mice were as anergic and suppressive as freshly isolated BALB/c CD25⁺ cells (Fig. 8B). Donor-derived CD25⁺ cells from nonimmunized mice showed somewhat lower suppressor activity than donor-derived CD25⁺ cells from immunized mice, but remained completely unresponsive to stimulation. The CD25⁻ cells derived from CD25⁺ cells displayed even lower suppressor activity, but remained largely unresponsive to stimulation. Thus, down-regulation of CD25 expression in adoptively transferred CD25⁺ cells is associated with a reduction of regulatory activity. Immunization of the recipient mice maintains the suppressor activity of the donor-derived CD25⁺ population.

Foxp3 expression is independent of IL-2 signaling

IL-2^{-/-} mice develop lymphoproliferative diseases caused by defective T_{reg} development and/or function. We showed previously that T_{reg} cells do not need to produce IL-2, although their function is highly dependent on IL-2 signaling through CD25 expression. Moreover, the fact that splenic CD4⁺ T cells from IL-2^{-/-} mice displayed normal regulatory activity when transferred to IL-2-sufficient mice indicated that IL-2 was not necessary for the development of T_{reg} cells in the thymus (26).

To investigate the IL-2 dependence of CD4⁺CD25⁺ T cells, we isolated CD25⁺ and CD25⁻ from the thymus of IL-2^{-/-} and IL-2^{+/+} littermates. The proportion of CD4⁺CD8⁻CD25⁺ was only partially reduced in the thymus of IL-2^{-/-} mice and was greatly diminished in the spleen (Fig. 9A), in agreement with published

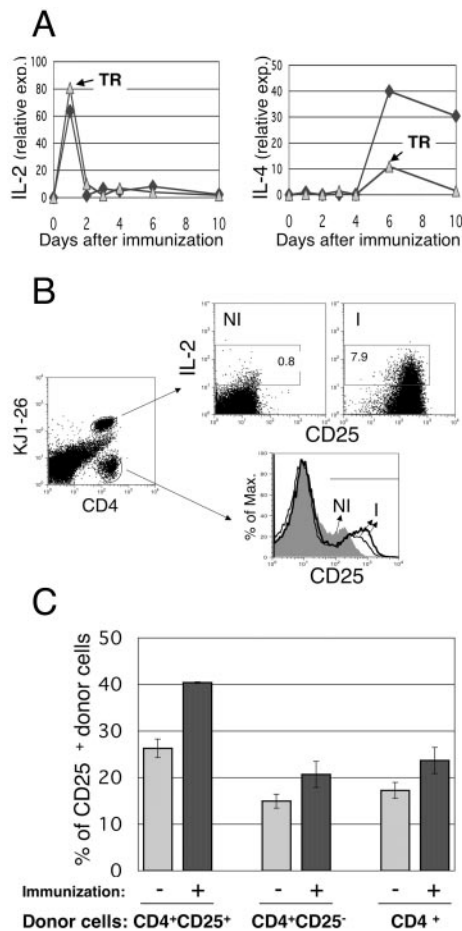


FIGURE 7. Immunization of T/B monoclonal mice with OVA-HA leads to IL-2 production by OVA-specific T cells and increased CD25 expression in T_{reg} cells. **A**, Kinetics of IL-2 and IL-4 production by endogenous OVA-specific T cells after immunization of T/B monoclonal mice. T/B monoclonal mice were transferred with 3×10^7 spleen cells from BALB/c mice. The transferred group and a nontransferred control group were immunized with OVA-HA in alum 1 day after transfer. On the indicated days after immunization, groups of three or four mice per group were killed, and OVA-specific T cells (KJ1-26⁺) were purified by magnetic sorting from the spleens of pooled group samples. After RNA extraction and cDNA synthesis, IL-2 and IL-4 expression were determined by real-time PCR. The RNA experiment is representative of two performed and is supported by extensive analysis of KJ1-26⁺ cells activation after immunization. **B**, T/B monoclonal mice were transferred with spleen cells of BALB/c mice as described in **A** and immunized with OVA-HA 20 days later. Two mice per group were killed 14 h after immunization. Spleen cells were harvested; surface-stained with anti-CD4, anti-CD25, and KJ1-26 Abs; subsequently fixed; permeabilized; and stained with anti-IL-2 Abs, without ex vivo PMA/ionomycin treatment. The dot plot on the left shows the gates for donor-derived (CD4⁺KJ1-26⁺) and endogenous cells (CD4⁺KJ1-26⁻). I, cells from immunized mice; NI, cells from nonimmunized mice. IL-2 production and CD25 expression by endogenous cells are shown in the upper middle and right plots. The histogram at the bottom shows the overlapping curves of CD25 expression by donor-derived cells from nonimmunized (solid gray) and two immunized mice samples (line). Very few IL-2-producing cells (1–2%) were detected in the donor-derived CD4⁺KJ1-26⁻ cells analyzed ex vivo at this time point (3 wk after transfer). **C**, Immunization of the recipient T/B monoclonal mice with OVA-HA partially rescues CD25 expression in transferred CD25⁺ T_{reg} cells. CD4⁺CD25⁺, CD4⁺CD25⁻, and total CD4⁺ cells were purified from spleens of BALB/c mice and transferred to T/B monoclonal mice (1×10^6 cells/mouse). Half of each group was immunized with OVA-HA. CD25 expression was analyzed in peripheral blood of T/B monoclonal mice 9 days after transfer. Shown are the percentages of CD25⁺ cells in gated donor-derived CD4⁺KJ1-26⁻ cells (average \pm SD; $n = 3$).

results (37, 38). Importantly, quantitative RNA expression analysis of purified populations of CD25⁺ cells from thymus or spleen of young IL-2^{-/-} mice demonstrated high levels of expression of Foxp3, albeit somewhat lower than those in the IL-2⁺ littermates (Fig. 9B). These results indicate that the thymic generation of a Foxp3⁺CD4⁺CD25⁺ population is only marginally impaired in IL-2^{-/-} mice, supporting our assertion that IL-2 is not required for the thymic development of T_{reg} , but is crucial at later stages. Moreover, our results show that the induction of Foxp3 expression is independent of IL-2 signaling.

Discussion

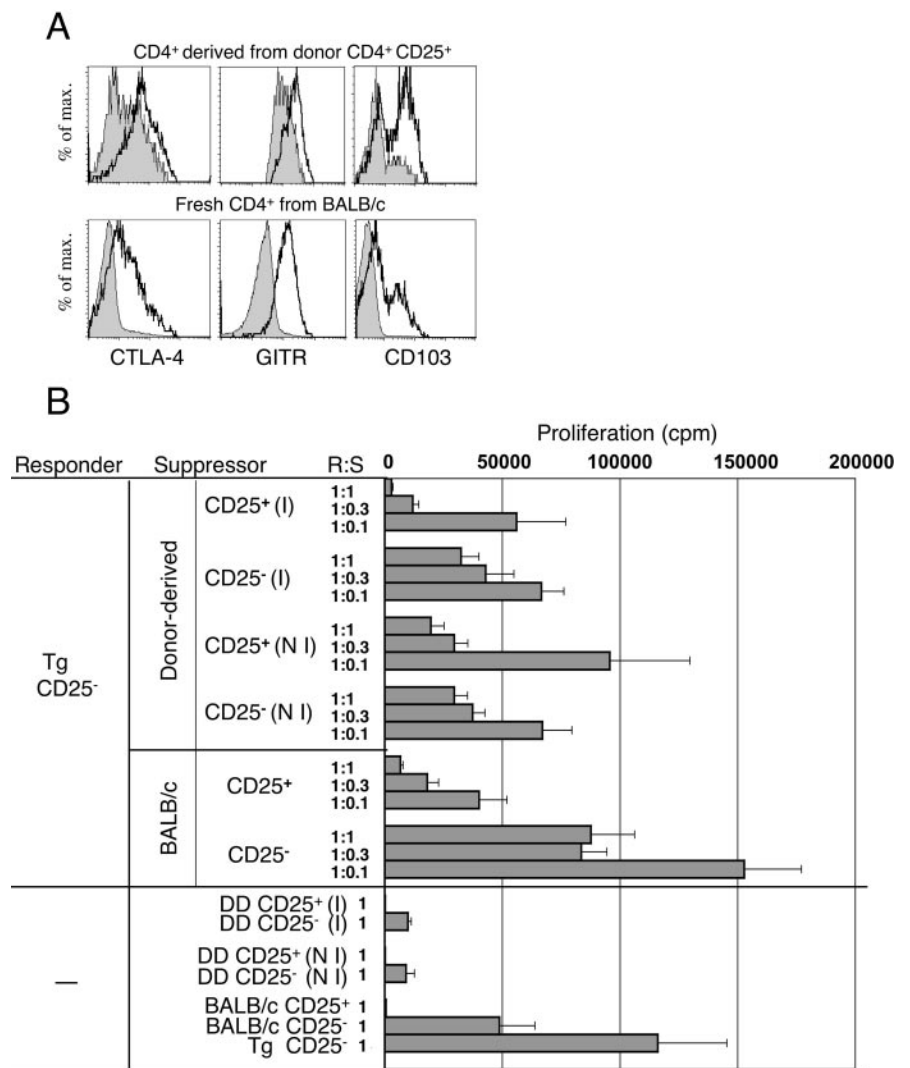
In this manuscript we showed that upon adoptive transfer of CD4⁺CD25⁻ T cells, a considerable proportion of the donor cells converted to CD25⁺ in vivo. We showed that this conversion is not the result of the outgrowth of a contaminant CD25⁺ population, and that the CD25⁻-derived CD25⁺ T cells are, phenotypically and functionally, bona fide T_{reg} cells. This conversion took place efficiently in animals that allow homeostatic proliferation of T cells, such as RAG-1^{-/-}, T/B monoclonal, or wild-type neonatal mice. In adult wild-type mice the conversion was much less efficient, although it could also be observed.

A number of recent studies reported the peripheral induction of T_{reg} cells using a variety of protocols (3, 39–51). Some of these induced T_{reg} cells are alloantigen-specific or foreign Ag-specific. The degree of phenotypic and functional resemblance between the induced regulatory cells and naturally occurring CD4⁺CD25⁺ T_{reg} cells was investigated to different degrees in the different studies. Some of the protocols led to T_{reg} cells that displayed phenotypes different from naturally occurring CD4⁺CD25⁺ T_{reg} cells (42, 52, 53), whereas other protocols led to cells indistinguishable from naturally occurring T_{reg} cells (49). The CD4⁺CD25⁺ T_{reg} cells generated through homeostatic proliferation of CD4⁺CD25⁻ T cells also behaved like naturally occurring T_{reg} cells in all parameters that we studied.

The CD25⁺ T cell-spiking experiments (Fig. 2B) demonstrated that T_{reg} cells generated through homeostatic proliferation do not represent the expansion of pre-existing CD4⁺CD25⁺ cells. On the basis that <1% of sorted human CD25⁺ cells remained alive by day 10 after activation, Walker et al. (40) concluded that the CD25⁻-derived CD25⁺ cells they obtained in vitro are not the product of the expansion of pre-existing CD25⁺ cells. In some cases, CD25⁻-cell-derived CD25⁺ T_{reg} cells were obtained from TCR-transgenic SCID or RAG^{-/-} mice (41, 49, 54). Because these mice contain virtually no CD25⁺ T cells before T_{reg} induction, outgrowth of pre-existing CD25⁺ cells is highly unlikely. Similarly, Ab depletion of CD25⁺ cells was used to show that T_{reg} cells were derived from CD25⁻ cells (43, 47, 48). In these three manuscripts it was shown or mentioned that few CD25⁺ cells were left behind after depletion, making it unlikely that contaminant CD25⁺ cells would play a role, although this possibility was not formally excluded.

CD4⁺CD25⁺Foxp3⁺ T_{reg} cells could be derived from CD25⁻ T cells in vitro by TCR stimulation in the presence of TGF- β (39, 47). Interestingly, the treatment that induced the conversion of CD25⁻ cells to CD25⁺ T_{reg} cells did not trigger expansion of pre-existing CD4⁺CD25⁺ cells (39). Thus, in this case the CD25⁺ T_{reg} cells also do not appear to be the product of outgrowth of pre-existing CD25⁺ cells. In contrast, Horwitz et al. (55) reported that TGF- β induction of human CD4⁺CD25⁺ T cells derived in vitro from naive CD45RA⁺RO⁻ T cells was markedly decreased if a pre-existing population (1% of naive CD4⁺ T cells) expressing CD25 was depleted. A subsequent study by the same group concluded that the residual CD25⁺ T cells greatly increase the number

FIGURE 8. Loss of CD25 expression in T_{reg} cells during homeostatic proliferation is accompanied by a partial loss of regulatory function. **A**, Decreased expression of T_{reg}-associated genes in CD25⁻ cells derived from CD25⁺ donor cells. CD4⁺CD25⁺ cells were purified from normal BALB/c mice and transferred to T/B monoclonal mice. One day later, half the recipient mice were immunized with OVA-HA. Two weeks after transfer, the mice were killed, spleen cells were harvested, and the expression of Treg-associated proteins, CTLA-4, CD103, and GITR, in the donor-derived population was determined by flow cytometry. The figure shows overlapping histograms of gated CD25⁺KJ1-26⁻ (line) and CD25⁻KJ1-26⁻ (solid) donor-derived CD4⁺ cells. The expressions of CTLA-4, CD103, and GITR in fresh spleen cells from a BALB/c mouse are shown for comparison. Overlapping histograms of CD4⁺CD25⁺ (line) and CD4⁺CD25⁻ (solid) BALB/c cells are shown. **B**, Partial loss of suppressor activity in CD25⁻ cells derived from CD25⁺ donor cells. The ability of donor-derived CD4⁺CD25⁺ and CD4⁺CD25⁻ cells to respond to anti-CD3 stimulation and to exert suppression was tested in vitro as described in Fig. 3C. Donor-derived CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were purified from T/B monoclonal mice that were transferred with BALB/c CD4⁺CD25⁺ cells and were immunized (I) or not immunized (NI). Responder cells were DO11.10 RAG-1^{-/-} cells (Tg CD25⁻). Donor-derived cells as well as freshly isolated BALB/c CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were cultured alone or were cocultured with responder cells in the presence of anti-CD3 Abs and APCs. Ratios of responders to suppressors were 1:1, 1:0.3, and 1:0.1. Proliferation was measured on the third day of culture by [³H]thymidine incorporation. Results are the average and SD of triplicate cultures. DD, donor-derived.



of CD25⁻ T cells that become CD25⁺ (44). Using a TGF- β -inducible system, Peng et al. (46) showed that TGF- β promotes expansion of the CD4⁺CD25⁺Foxp3⁺ T_{reg} pool, although conversion of CD25⁻ cells was not studied in this system.

Our results have several implications. First, homeostatic proliferation of lymphocytes is believed to play an important role in the immune systems of young (newborn) and aging normal individuals. In addition, several cancer treatments cause partial lymphocyte ablations that trigger homeostatic proliferation, and chronic infections can alter thymic output and cause homeostatic proliferation. Low level homeostatic proliferation is likely to be continuously taking place. Thus, in vivo conversion of CD4⁺CD25⁻ T cells into CD4⁺CD25⁺ T_{reg} cells under homeostatic proliferation conditions could play an important balancing role in the immune system.

Although naive CD25⁻ T cells can eventually give rise to CD25⁺ T_{reg} via homeostatic proliferation (Fig. 5), the capacity of CD25⁻ cells to generate CD25⁺ T_{reg} cells is not limitless. Re-transfer experiments in which CD25⁻ T cells were injected, and the donor-derived CD25⁻ cells were purified and reinjected into secondary recipients showed a 5-fold decrease in the fraction of secondary donor-derived CD25⁺ cells compared with the fraction of CD25⁺ cells that arises upon transfer of fresh CD25⁻ T cells (data not shown). Although fewer CD25⁺ cells were generated upon these retransfers, the CD25⁺ cells displayed normal T_{reg} properties, such as elevated expression of Foxp3 (data not shown).

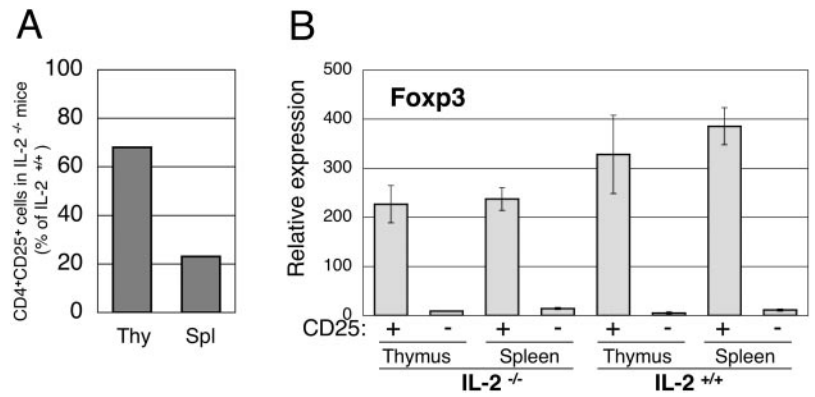
Therefore, it appears that the generation of CD25⁺ cells is not a stochastic event by which any CD25⁻ T cell has a given probability of becoming CD25⁺. Rather, many CD25⁻ cells are, seemingly, not able to generate CD25⁺ cells in these experimental conditions. These data are consistent with a crucial role of TCR specificity in the generation of T_{reg} cells, whereby only a fraction of CD25⁻ T cells would express TCR chains that are compatible with T_{reg} generation under particular stimuli (19, 56).

Another implication of the in vivo generation of CD4⁺CD25⁺ T_{reg} cells from CD4⁺CD25⁻ cells is that, considered together with the inability of genetically deficient CD25^{-/-} T cells to exert suppression (26), it provides a model to explain the protective activity of CD4⁺CD25⁻ T cells in models of autoimmunity and allergy; the ability of CD4⁺CD25⁻ cells to protect in these disease models would depend on the generation of CD25⁺ T_{reg} cells in the periphery.

In this manuscript we also determined that acquisition/loss of CD25 expression correlated with the acquisition/loss of suppressor phenotype, and found that IL-2, produced by cells other than T_{reg}, is one of the key factors in determining the maintenance of CD25 expression. Cells that do not produce IL-2, such as nonexpanding CD25⁻ T cells or IL-2^{-/-} T cells, do not support CD25 expression.

In vitro, CD4⁺CD25⁺ T_{reg} cells exert their suppressive activity by preventing IL-2 production by effector cells (57, 58); however,

FIGURE 9. CD4⁺CD8⁻CD25⁺Foxp3⁺ cells develop in the thymus of IL-2^{-/-} mice. **A**, The expression of CD25 in CD4⁺CD8⁻ single-positive cells from thymus and CD4⁺ cells from spleen of 3-wk-old IL-2^{-/-} and IL-2^{+/+} BALB/c littermates was determined by flow cytometry. The figure shows the CD25⁺ cells in IL-2^{-/-} mice as a percentage of the IL-2^{+/+} wild-type littermates (100%). **B**, CD25⁺ and CD25⁻ CD4⁺CD8⁻ cells were purified from thymus and spleen of 3-wk-old IL-2^{-/-} and IL-2^{+/+} littermates. The expression of Foxp3 in the samples was determined by real-time PCR.



the situation in vivo is quite different. Indeed, the early burst of IL-2 production by OVA-specific T cells upon immunization of T/B monoclonal mice is unaffected by the presence of T_{reg} cells (Fig. 7A) despite the striking suppressive effect of T_{reg} cells on IL-4 production and IgE switching (Fig. 7A) (24). Thus, as proposed by Furtado et al. (26), IL-2 produced by activated effector T cells fuels the peripheral conversion of CD25⁻ to CD25⁺ cells and sustains CD25 expression, thus establishing an autoregulatory loop during immune responses.

Our observations emphasize the interdependence of T_{reg} and conventional T cells to maintain a peripheral balanced immune system. Although CD25⁺ cells down-regulate immune responses, self or Ag-specific conventional T cells help maintain a functional T_{reg} compartment.

Acknowledgments

We thank Dan Littman, Shimon Sakaguchi, Shiqian Shen, and Daniel Mucida for comments and/or suggestions on the manuscript; Takeshi Egawa for help with the LSR-II (BD Biosciences); Marlin Camps-Ramírez for the CTLA-4 and GITR primers; and Fidel Zavala for the BALB/c Thy1.1 mice.

References

- Mason, D., and F. Powrie. 1998. Control of immune pathology by regulatory T cells. *Curr. Opin. Immunol.* 10:649.
- Maloy, K. J., and F. Powrie. 2001. Regulatory T cells in the control of immune pathology. *Nat. Immunol.* 2:816.
- Curotto de Lafaille, M. A., and J. J. Lafaille. 2002. CD4⁺ regulatory T cells in autoimmunity and allergy. *Curr. Opin. Immunol.* 14:771.
- Bluestone, J. A., and A. K. Abbas. 2003. Natural versus adaptive regulatory T cells. *Nat. Rev. Immunol.* 3:253.
- 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.
- 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.
- Shevach, E. M., R. S. McHugh, C. A. Piccirillo, and A. M. Thornton. 2001. Control of T-cell activation by CD4⁺ CD25⁺ suppressor T cells. *Immunol. Rev.* 182:58.
- Shevach, E. M. 2002. CD4⁺ CD25⁺ suppressor T cells: more questions than answers. *Nat. Rev. Immunol.* 2:389.
- Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299:1057.
- 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.
- Khattri, R., T. Cox, S. A. Yasayko, and F. Ramsdell. 2003. An essential role for Scurfin in CD4⁺CD25⁺ T regulatory cells. *Nat. Immunol.* 4:337.
- Read, S., V. Malmstrom, and F. Powrie. 2000. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25⁺CD4⁺ regulatory cells that control intestinal inflammation. *J. Exp. Med.* 192:295.
- Takahashi, T., T. Tagami, S. Yamazaki, T. Uede, J. Shimizu, N. Sakaguchi, T. W. Mak, and S. Sakaguchi. 2000. Immunologic self-tolerance maintained by CD25⁺CD4⁺ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J. Exp. Med.* 192:303.

- Bennett, C. L., J. Christie, F. Ramsdell, M. E. Brunkow, P. J. Ferguson, L. Whitesell, T. E. Kelly, F. T. Saulsbury, P. F. Chance, and H. D. Ochs. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat. Genet.* 27:20.
- Brunkow, M. E., E. W. Jeffery, K. A. Hjerrild, B. Paepfer, L. B. Clark, S. A. Yasayko, J. E. Wilkinson, D. Galas, S. F. Ziegler, and F. Ramsdell. 2001. Disruption of a new forkhead/winged-helix protein, scurf, results in the fatal lymphoproliferative disorder of the scurf mouse. *Nat. Genet.* 27:68.
- Chatila, T. A., F. Blaeser, N. Ho, H. M. Lederman, C. Voulgaropoulos, C. Helms, and A. M. Bowcock. 2000. JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic dysregulation syndrome. *J. Clin. Invest.* 106:R75.
- Tivol, E. A., F. Borriello, A. N. Schweitzer, W. P. Lynch, J. A. Bluestone, and A. H. Sharpe. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 3:541.
- Waterhouse, P., J. M. Penninger, E. Timms, A. Wakeham, A. Shahinian, K. P. Lee, C. B. Thompson, H. Griesser, and T. W. Mak. 1995. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science* 270:985.
- Olivares-Villagomez, D., A. K. Wensky, Y. Wang, and J. J. Lafaille. 2000. Repertoire requirements of CD4⁺ T cells that prevent spontaneous autoimmune encephalomyelitis. *J. Immunol.* 164:5499.
- Furtado, G. C., D. Olivares-Villagomez, M. A. Curotto de Lafaille, A. K. Wensky, J. A. Latkowski, and J. J. Lafaille. 2001. Regulatory T cells in spontaneous autoimmune encephalomyelitis. *Immunol. Rev.* 182:122.
- Stephens, L. A., and D. Mason. 2000. CD25 is a marker for CD4⁺ thymocytes that prevent autoimmune diabetes in rats, but peripheral T cells with this function are found in both CD25⁺ and CD25⁻ subpopulations. *J. Immunol.* 165:3105.
- Gonzalez, A., I. Andre-Schmutz, C. Carnaud, D. Mathis, and C. Benoist. 2001. Damage control, rather than unresponsiveness, effected by protective DX5⁺ T cells in autoimmune diabetes. *Nat. Immunol.* 2:1117.
- Apostolou, I., A. Sarukhan, L. Klein, and H. Von Boehmer. 2002. Origin of regulatory T cells with known specificity for antigen. *Nat. Immunol.* 3:756.
- Curotto de Lafaille, M. A., S. Muriglan, M. J. Sunshine, Y. Lei, N. Kutchukhidze, G. C. Furtado, A. K. Wensky, D. Olivares-Villagomez, and J. J. Lafaille. 2001. Hyperimmunoglobulin E response in mice with monoclonal populations of B and T lymphocytes. *J. Exp. Med.* 194:1349.
- Annacker, O., R. Pimenta-Araujo, O. Buren-Defranoux, T. C. Barbosa, A. Cumano, and A. Bandeira. 2001. CD25⁺ CD4⁺ T cells regulate the expansion of peripheral CD4 T cells through the production of IL-10. *J. Immunol.* 166:3008.
- Furtado, G. C., M. A. Curotto de Lafaille, N. Kutchukhidze, and J. J. Lafaille. 2002. Interleukin 2 signaling is required for CD4⁺ regulatory T cell function. *J. Exp. Med.* 196:851.
- Gavin, M. A., S. R. Clarke, E. Negrou, A. Gallegos, and A. Rudensky. 2002. Homeostasis and anergy of CD4⁺CD25⁺ suppressor T cells in vivo. *Nat. Immunol.* 3:33.
- Almeida, A. R., N. Legrand, M. Papiernik, and A. A. Freitas. 2002. Homeostasis of peripheral CD4⁺ T cells: IL-2R α and IL-2 shape a population of regulatory cells that controls CD4⁺ T cell numbers. *J. Immunol.* 169:4850.
- Klein, L., K. Khazaie, and H. von Boehmer. 2003. In vivo dynamics of antigen-specific regulatory T cells not predicted from behavior in vitro. *Proc. Natl. Acad. Sci. USA* 100:8886.
- Laurie, K. L., I. R. Van Driel, and P. A. Gleeson. 2002. The role of CD4⁺CD25⁺ immunoregulatory T cells in the induction of autoimmune gastritis. *Immunol. Cell Biol.* 80:567.
- McHugh, R. S., M. J. Whitters, C. A. Piccirillo, D. A. Young, E. M. Shevach, M. Collins, and M. C. Byrne. 2002. CD4⁺CD25⁺ immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* 16:311.
- Shimizu, J., S. Yamazaki, T. Takahashi, Y. Ishida, and S. Sakaguchi. 2002. Stimulation of CD25⁺CD4⁺ regulatory T cells through GITR breaks immunological self-tolerance. *Nat. Immunol.* 22:22.
- Zelenika, D., E. Adams, S. Humm, L. Graca, S. Thompson, S. P. Cobbold, and H. Waldmann. 2002. Regulatory T cells overexpress a subset of Th2 gene transcripts. *J. Immunol.* 168:1069.

34. Lehmann, J., J. Huehn, M. de la Rosa, F. Maszyzna, U. Kretschmer, V. Krenn, M. Brunner, A. Scheffold, and A. Hamann. 2002. Expression of the integrin $\alpha_E\beta_7$ identifies unique subsets of CD25⁺ as well as CD25⁻ regulatory T cells. *Proc. Natl. Acad. Sci. USA* 99:13031.
35. Min, B., R. McHugh, G. D. Sempowski, C. Mackall, G. Foucras, and W. E. Paul. 2003. Neonates support lymphopenia-induced proliferation. *Immunity* 18:131.
36. Khoruts, A., A. Mondino, K. A. Pape, S. L. Reiner, and M. K. Jenkins. 1998. A natural immunological adjuvant enhances T cell clonal expansion through a CD28-dependent, interleukin (IL)-2-independent mechanism. *J. Exp. Med.* 187:225.
37. Papiernik, M., M. L. de Moraes, C. Pontoux, F. Vasseur, and C. Penit. 1998. Regulatory CD4 T cells: expression of IL-2R α chain, resistance to clonal deletion and IL-2 dependency. *Int. Immunol.* 10:371.
38. Antov, A., L. Yang, M. Vig, D. Baltimore, and L. Van Parijs. 2003. Essential role for STAT5 signaling in CD25⁺CD4⁺ regulatory T cell homeostasis and the maintenance of self-tolerance. *J. Immunol.* 171:3435.
39. Chen, W., W. Jin, N. Hardegen, K. J. Lei, L. Li, N. Marinos, G. McGrady, and S. M. Wahl. 2003. Conversion of peripheral CD4⁺CD25⁻ naive T cells to CD4⁺CD25⁺ regulatory T cells by TGF- β induction of transcription factor Foxp3. *J. Exp. Med.* 198:1875.
40. Walker, M. R., D. J. Kasprovicz, 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.
41. Cobbold, S. P., R. Castejon, E. Adams, D. Zelenika, L. Graca, S. Humm, and H. Waldmann. 2004. Induction of foxP3⁺ regulatory T cells in the periphery of T cell receptor transgenic mice tolerized to transplants. *J. Immunol.* 172:6003.
42. Chen, T. C., S. P. Cobbold, P. J. Fairchild, and H. Waldmann. 2004. Generation of anergic and regulatory T cells following prolonged exposure to a harmless antigen. *J. Immunol.* 172:5900.
43. Karim, M., C. I. Kingsley, A. R. Bushell, B. S. Sawitzki, and K. J. Wood. 2004. Alloantigen-induced CD25⁺CD4⁺ regulatory T cells can develop in vivo from CD25⁻CD4⁺ precursors in a thymus-independent process. *J. Immunol.* 172:923.
44. Zheng, S. G., J. H. Wang, J. D. Gray, H. Soucier, and D. A. Horwitz. 2004. Natural and induced CD4⁺CD25⁺ cells educate CD4⁺CD25⁻ cells to develop suppressive activity: the role of IL-2, TGF- β , and IL-10. *J. Immunol.* 172:5213.
45. Fantini, M. C., C. Becker, G. Monteleone, F. Pallone, P. R. Galle, and M. F. Neurath. 2004. Cutting edge: TGF- β induces a regulatory phenotype in CD4⁺CD25⁻ T cells through Foxp3 induction and down-regulation of Smad7. *J. Immunol.* 172:5149.
46. Peng, Y., Y. Laouar, M. O. Li, E. A. Green, and R. A. Flavell. 2004. TGF- β regulates in vivo expansion of Foxp3-expressing CD4⁺CD25⁺ regulatory T cells responsible for protection against diabetes. *Proc. Natl. Acad. Sci. USA* 101:4572.
47. Park, H. B., D. J. Paik, E. Jang, S. Hong, and J. Youn. 2004. Acquisition of anergic and suppressive activities in transforming growth factor- β -costimulated CD4⁺CD25⁻ T cells. *Int. Immunol.* 16:1203.
48. Verhasselt, V., O. Vosters, C. Beuneu, C. Nicaise, P. Stordeur, and M. Goldman. 2004. Induction of FOXP3-expressing regulatory CD4^{pos} T cells by human mature autologous dendritic cells. *Eur. J. Immunol.* 34:762.
49. Apostolou, I., and H. Von Boehmer. 2004. In vivo instruction of suppressor commitment in naive T cells. *J. Exp. Med.* 199:1401.
50. Chen, C., W. H. Lee, P. Yun, P. Snow, and C. P. Liu. 2003. Induction of autoantigen-specific Th2 and Tr1 regulatory T cells and modulation of autoimmune diabetes. *J. Immunol.* 171:733.
51. Bynoe, M. S., J. T. Evans, C. Viret, and C. A. Janeway, Jr. 2003. Epicutaneous immunization with autoantigenic peptides induces T suppressor cells that prevent experimental allergic encephalomyelitis. *Immunity* 19:317.
52. Vieira, P. L., J. R. Christensen, S. Minaee, E. J. O'Neill, F. J. Barrat, A. Boonstra, T. Barthlott, B. Stockinger, D. C. Wraith, and A. O'Garra. 2004. IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4⁺CD25⁺ regulatory T cells. *J. Immunol.* 172:5986.
53. Feunou, P., L. Poulin, C. Habran, A. Le Moine, M. Goldman, and M. Y. Braun. 2003. CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells act respectively as inducer and effector T suppressor cells in superantigen-induced tolerance. *J. Immunol.* 171:3475.
54. Thorstenson, K. M., and A. Khoruts. 2001. Generation of anergic and potentially immunoregulatory CD25⁺CD4 T cells in vivo after induction of peripheral tolerance with intravenous or oral antigen. *J. Immunol.* 167:188.
55. Yamagiwa, S., J. D. Gray, S. Hashimoto, and D. A. Horwitz. 2001. A role for TGF- β in the generation and expansion of CD4⁺CD25⁺ regulatory T cells from human peripheral blood. *J. Immunol.* 166:7282.
56. Lafaille, J. J., K. Nagashima, M. Katsuki, and S. Tonegawa. 1994. High incidence of spontaneous autoimmune encephalomyelitis in immunodeficient anti-myelin basic protein T cell receptor transgenic mice. *Cell* 78:399.
57. Thornton, A. M., and E. M. Shevach. 1998. CD4⁺CD25⁺ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J. Exp. Med.* 188:287.
58. Thornton, A. M., and E. M. Shevach. 2000. Suppressor effector function of CD4⁺CD25⁺ immunoregulatory T cells is antigen nonspecific. *J. Immunol.* 164:183.