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$CD25^-$ T Cells Generate $CD25^+Foxp3^+$ Regulatory T Cells by Peripheral Expansion[1]

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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 encephalomylitis (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 functionality 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 $I\text{-}A^d$-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 $RAG\text{-}1^{-/-}$ 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 $RAG\text{-}1^{-/-}$) 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 $IL\text{-}2^{-/-}$ 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). TCR$\alpha\beta$-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).

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

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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 × 10^6 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 10^7 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, ACTGGGGTCTTCTCCCTCAA, CGTGGGAAGGTGCAGAG TAG; CTLA-4, GTTGGGGGCATTTTCACATA, TTTTACAGTTTCCT GGTCTC; and β-actin, TGACAGGATGCAGAAGGAGA, GTACTTG CGCTCAGGAGGAG.

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 × 10^4 responder cells, 2 × 10^4 APC (mitomycin C-treated spleen cells from TCR$\alpha\beta$-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 [^{3}H]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. 1*A*), labeled

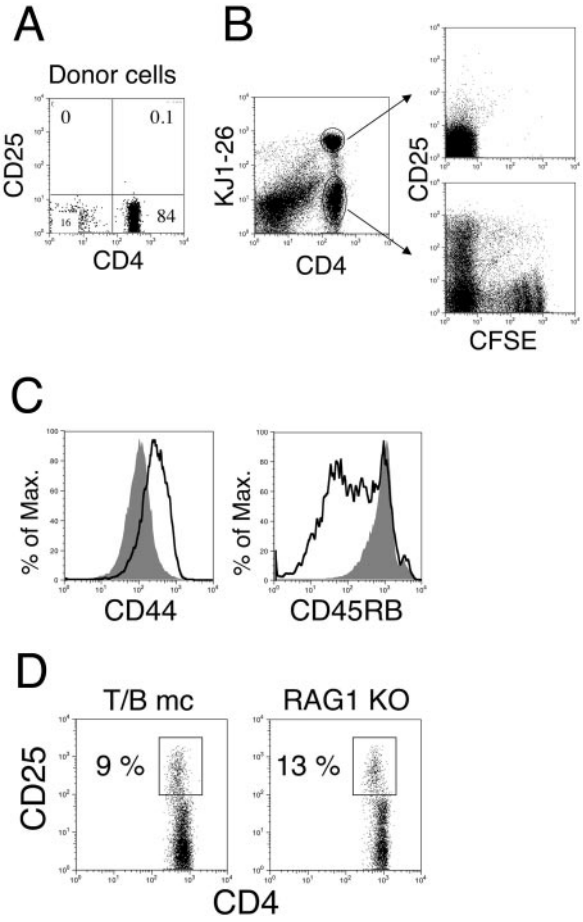

FIGURE 1. Conversion of $CD4^+CD25^-$ cells to $CD25^+$ cells in T/B monoclonal and RAG-$1^{-/-}$ recipient mice during peripheral expansion. *A*, $CD4^+CD25^-$ cells were purified from spleen and lymph nodes of BALB/c mice with ≤0.1% contamination by $CD25^+$ cells. The cells were labeled with CFSE and transferred to T/B monoclonal mice (5 × 10^6 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 × 10^6 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 $KJ1\text{-}26^-CD4^+$ in Fig. 1*B*). Cells that became $CD25^+$ were found in the population that underwent extensive proliferation (Fig. 1*B*). 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. 1*C*). In contrast, the endogenous OVA-specific $KJ1\text{-}26^+CD4^+$ 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. 1*D*).

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. 2*A*). Upon injection of $CD4^+CD25^-$ cells obtained from secondary lymphoid organs (designated peripheral in Fig. 2*A*) or $CD4^+CD25^-$ single-positive cells from thymus (designated SP in Fig. 2*A*), 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. 2*A*). 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. 2*B*). 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 Vα and Vβ 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. 3*A*). 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. 3*B*). Thus, phenotypically, $CD4^+CD25^+$ cells derived from homeostatic expansion of $CD4^+CD25^-$ cells are very similar to freshly isolated $CD4^+CD25^+$ T cells.

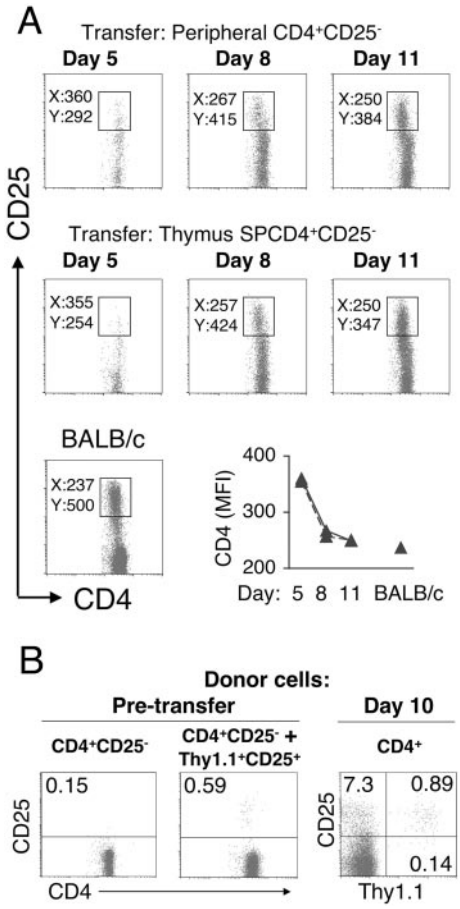

FIGURE 2. *A*, Kinetics of conversion of $CD25^-$ cells into $CD25^{high}CD4^{low}$ cells. Purified $CD4^+CD25^-$ cells from spleen and lymph nodes (peripheral) or from thymus of BALB/c mice were transferred to T/B monoclonal mice (2×10^6 cells/mouse). The expression of CD25 in donor-derived $CD4^+$ 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. 3*C*). Little or no suppressor activity was detected in the donor-derived $CD25^-$ population.

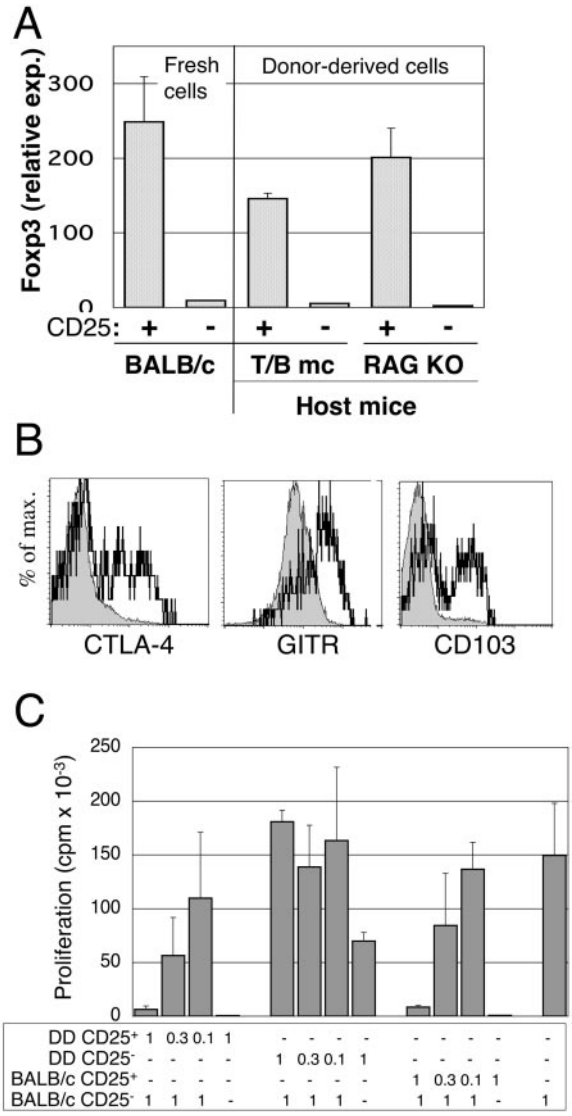

FIGURE 3. $CD4^+CD25^+$ cells derived from $CD4^+CD25^-$ cells have phenotypic and functional characteristics of T_{reg} cells. *A*, $CD25^+$ cells derived from $CD25^-$ cells express Foxp3 mRNA. $CD4^+CD25^-$ cells were purified from spleen and lymph nodes of BALB/c mice and transferred into T/B monoclonal 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 Foxp3 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 ($CD4^+KJ1\text{-}26^-$) was determined by flow cytometry. The figure shows histograms of gated $CD4^+KJ1\text{-}26^-CD25^+$ (line) and $CD4^+KJ1\text{-}26^-CD25^-$ (▨) 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 [^{3}H]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.

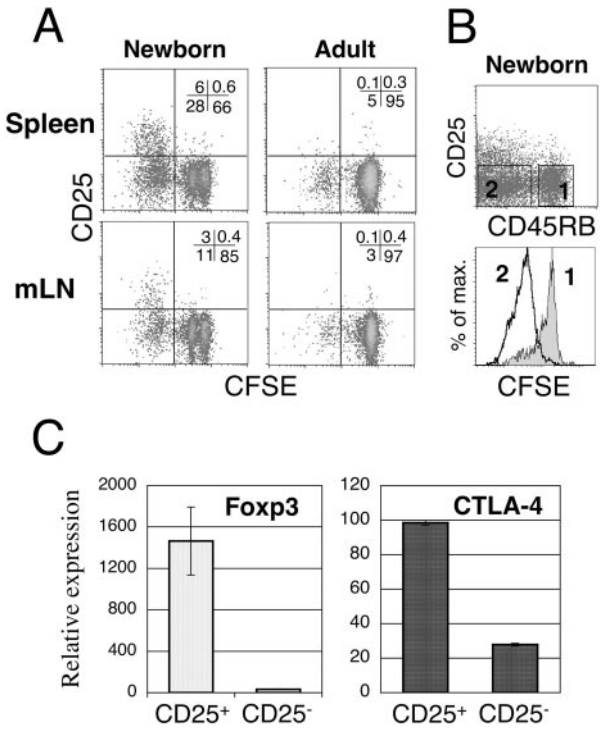

FIGURE 4. $CD4^+CD25^-$ cells transferred into wild-type BALB/c neonates give rise to $CD4^+CD25^+CD45RB^{low}$ cells. $CD4^+CD25^-$ cells were purified from spleen and lymph nodes of BALB/c Thy1.1/1.2 mice, labeled with CFSE, and transferred into neonates (0.5–1 $\times$ 10^6/mouse) or adult (5×10^6 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 $CD25^+Foxp3^+$ 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 Foxp3 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. 4*A*). 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. 4*B*). 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. 4*C*). 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. 5*A*). However, 21 days after transfer, $CD25^+$ cells derived from $CD25^-RB^{high}$ or RB^{low} cells had similar FACS profiles (Fig. 5*A*). 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. 5*B*). 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.

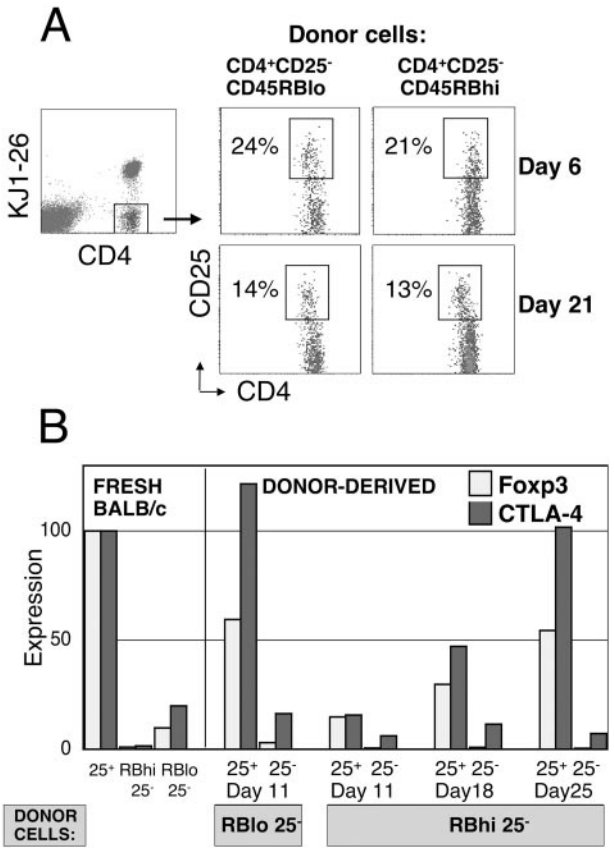

FIGURE 5. $CD4^+CD25^-CD45RB^{high}$ naive T cells give raise to T_{reg} cells in vivo with delayed kinetics. *A*, $CD4^+CD25^-CD45RB^{low}$ (RBlo) and $CD4^+CD25^-CD45RB^{high}$ (RBhi) T cells were purified from BALB/c mice and transferred to T/B monoclonal mice (1×10^6 cells/mouse). The expression of CD25 in donor-derived cells ($CD4^+$ $KJ1\text{-}26^-$) 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 $RAG\text{-}1^{-/-}$ mice were used as recipients.

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

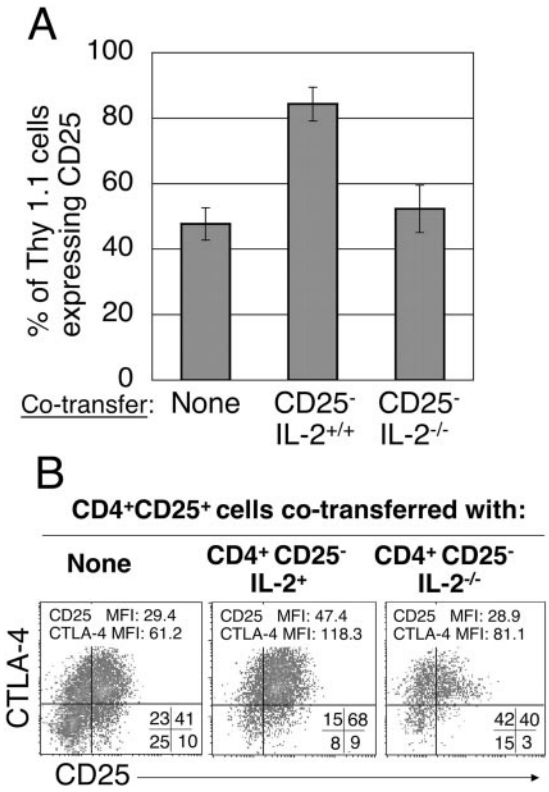

FIGURE 6. Wild-type $CD4^+CD25^-$ cells, but not IL-2-deficient $CD4^+CD25^-$ cells, help maintain a functional population of peripheral $CD4^+CD25^+$ T_{reg} cells. *A*, $CD4^+CD25^+$ cells from BALB/c Thy1.1 mice were transferred alone (0.5×10^6), or cotransferred with 2×10^6 $CD4^+CD25^-$ cells from 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. 7*A*). 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. 7*B*). 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. 7*B*, MFI of nonimunized, 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. 8*A*). 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. 8*B*). 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. 9*A*), in agreement with published

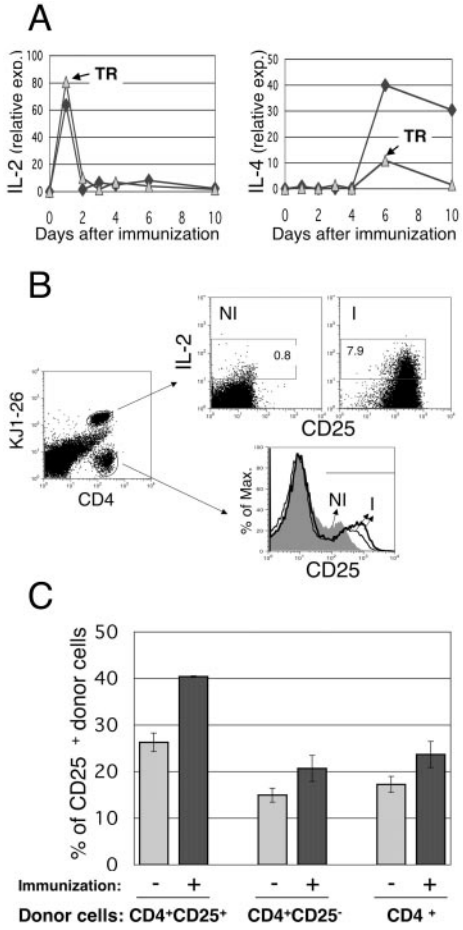

FIGURE 7. Immunization of T/B monoclonal mice with OVA-HA leads to IL-2 production by OVA-specific T cells and increased CD25 expression in T_{reg} cells. *A*, Kinetics of IL-2 and IL-4 production by endogenous OVA-specific T cells after immunization of T/B monoclonal mice. T/B monoclonal mice were transferred 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. 9*B*). 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, bonafide 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. 2*B*) 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

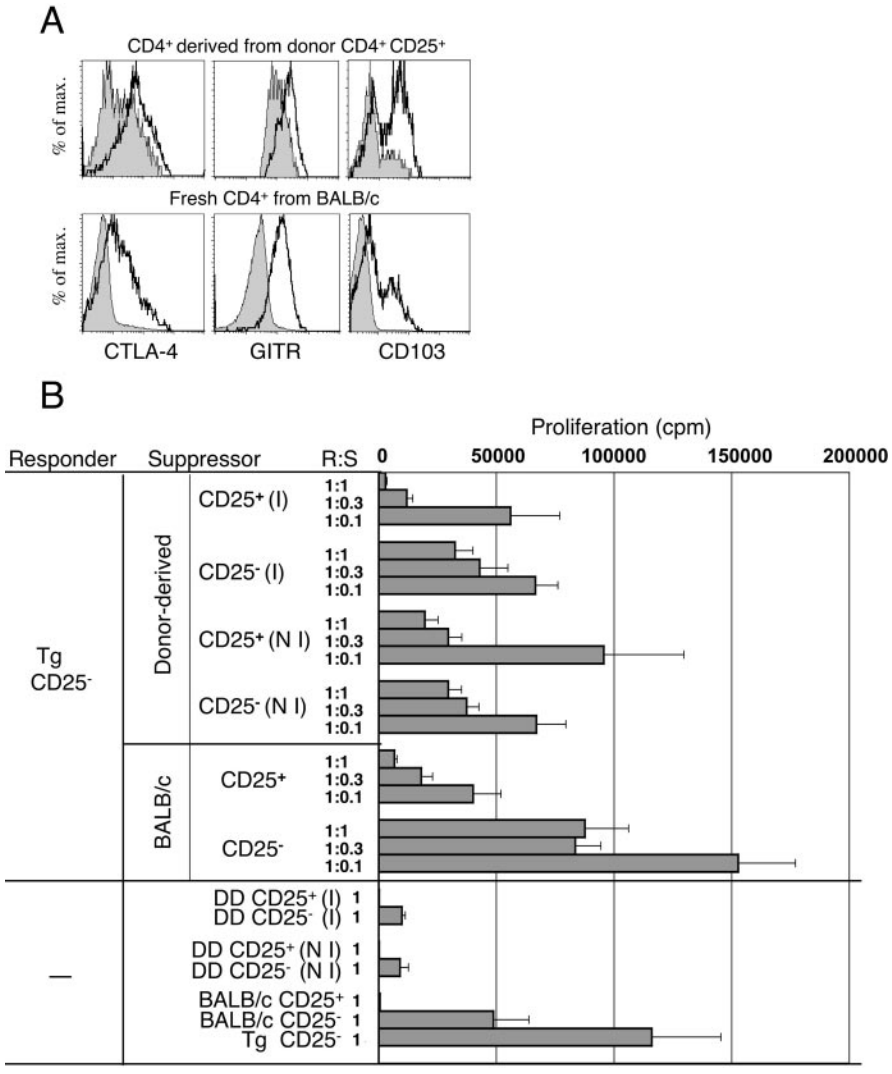

FIGURE 8. Loss of CD25 expression in T_{reg} cells during homeostatic proliferation is accompanied by a partial loss of regulatory function. *A*, Decreased expression of T_{reg}-associated genes in $CD25^-$ cells derived from $CD25^+$ donor cells. $CD4^+CD25^+$ cells were purified from normal BALB/c 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 $CD25^+KJ1\text{-}26^-$ (line) and $CD25^-KJ1\text{-}26^-$ (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. 3*C*. 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 [^{3}H]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. Retransfer 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,

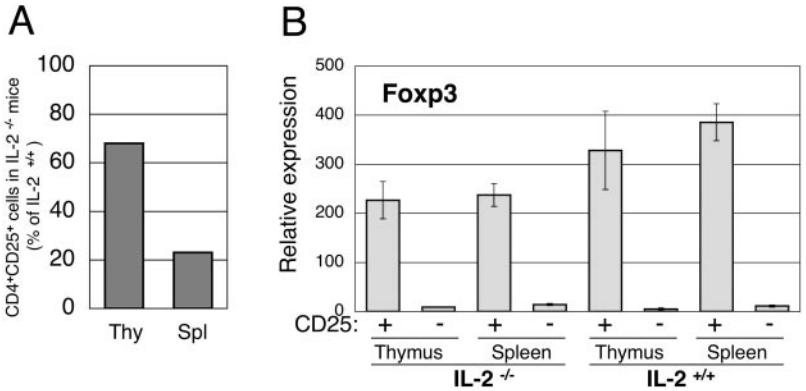

FIGURE 9. $CD4^+CD8^-CD25^+Foxp3^+$ cells develop in the thymus of IL-$2^{-/-}$ mice. *A*, The expression of CD25 in $CD4^+CD8^-$ single-positive cells from thymus and $CD4^+$ cells from spleen of 3-wk-old IL-$2^{-/-}$ and IL-2^+ BALB/c littermates was determined by flow cytometry. The figure shows the $CD25^+$ 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. 7*A*) despite the striking suppressive effect of T_{reg} cells on IL-4 production and IgE switching (Fig. 7*A*) (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

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References

1. Mason, D., and F. Powrie. 1998. Control of immune pathology by regulatory T cells. *Curr. Opin. Immunol. 10:649*.
2. Maloy, K. J., and F. Powrie. 2001. Regulatory T cells in the control of immune pathology. *Nat. Immunol. 2:816*.
3. Curotto de Lafaille, M. A., and J.J. Lafaille. 2002. $CD4^+$ regulatory T cells in autoimmunity and allergy. *Curr. Opin. Immunol. 14:771*.
4. Bluestone, J. A., and A.K. Abbas. 2003. Natural versus adaptive regulatory T cells. *Nat. Rev. Immunol. 3:253*.
5. 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*.
6. 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*.
7. 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*.
8. Shevach, E. M. 2002. $CD4^+$ $CD25^+$ suppressor T cells: more questions than answers. *Nat. Rev. Immunol. 2:389*.
9. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science 299:1057*.
10. 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*.
11. 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*.
12. 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*.
13. 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*.
14. 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*.
15. Brunkow, M. E., E. W. Jeffery, K. A. Hjerrild, B. Paeper, 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, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat. Genet. 27:68*.
16. 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*.
17. 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*.
18. 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*.
19. 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*.
20. 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*.
21. 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*.
22. 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*.
23. 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*.
24. 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. Hyper immunoglobulin E response in mice with monoclonal populations of B and T lymphocytes. *J. Exp. Med. 194:1349*.
25. Annacker, O., R. Pimenta-Araujo, O. Burlen-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*.
26. 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*.
27. 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*.
28. Almeida, A. R., N. Legrand, M. Papiernik, and A. A. Freitas. 2002. Homeostasis of peripheral $CD4^+$ T cells: IL-2Rα and IL-2 shape a population of regulatory cells that controls $CD4^+$ T cell numbers. *J. Immunol. 169:4850*.
29. 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*.
30. 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*.
31. 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*.
32. 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*.
33. 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. Maszyna, 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-2Rα 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. 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*.
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*.