# Conversion of $CD4^+$ $CD25^-$ cells into $CD4^+$ $CD25^+$ regulatory T cells in vivo requires B7 costimulation, but not the thymus

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**The $CD4^+$ $CD25^+$ regulatory T cells play a critical role in controlling autoimmunity, but little is known about their development and maintenance. In this study, we investigated whether $CD4^+$ $CD25^-$ cells can convert to $CD4^+$ $CD25^+$ regulatory T cells in vivo under natural conditions. $CD4^+$ $CD25^-$ cells from $CD45.1^+$ mice were sorted and transferred into congenic $CD45.2^+$ mice. Converted $CD4^+$ $CD25^+$ cells could be detected in lymphoid organs as early as 1 wk after transfer and by 6 wk after transfer, 5–12% of transferred $CD4^+$ cells expressed CD25. Converted $CD4^+$ $CD25^+$ cells themselves failed to proliferate after stimulation, but could suppress proliferation of responder cells in vitro, and also expressed high levels of Foxp3 mRNA. In addition, $CD4^+$ $CD25^-$ cells transferred into thymectomized congenic mice converted to $CD4^+$ $CD25^+$ cells that also suppressed responder cell proliferation in vitro, and expressed high levels of Foxp3 mRNA. Finally, $CD4^+$ $CD25^-$ cells transferred into $B7^{-/-}$ mice failed to convert into $CD4^+$ $CD25^+$ cells that exhibit the regulatory phenotype. These data indicate that $CD4^+$ $CD25^-$ cells convert into $CD4^+$ $CD25^+$ regulatory T cells spontaneously in vivo and suggest that this conversion process could contribute significantly to the maintenance of the peripheral $CD4^+$ $CD25^+$ regulatory T cell population.**

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Abbreviations used: CFSE, carboxyfluorescein diacetate succinimidyl ester; CTLA-4, cytotoxic T lymphocyte–associated antigen 4; GITR, glucocorticoid-induced TNF receptor gene.

Regulating potentially autoreactive cells that have escaped negative selection in the thymus is an important function of peripheral tolerance. A growing body of evidence suggests that a population of regulatory T cells, $CD4^+$ $CD25^+$ T cells, first identified by Asano et al. (1) and Sakaguchi et al. (2), is critical for controlling a wide variety of immune responses including those that cause many types of autoimmune disease. Depletion of this population of cells results in multi-organ autoimmune diseases in a variety of strains of mice (1, 2). It is still unclear to date whether the $CD4^+$ $CD25^+$ regulatory T cell population represents a distinct lineage of T cells. Several lines of evidence suggest that these cells develop in the thymus. For example, $CD4^+$ ($CD8^-$) $CD25^+$ T cells can be found in the thymus and exhibit phenotypic and functional characteristics that are identical to those found in peripheral $CD4^+$ $CD25^+$ T cells. Adoptive transfer of this population of thymocytes prevents development of a variety of autoimmune and inflammatory diseases (3). Furthermore, $CD4^+$ $CD25^+$ T cells develop directly in fetal thymic organ cultures (3). These cells appear to be positively selected on thymic epithelium because mice that do not express MHC class II on their thymic cortical epithelium fail to develop $CD4^+$ $CD25^+$ regulatory T cells, and transgenic mice that express specific peptide on their thymic stromal cells produce extremely high percentages of $CD4^+$ $CD25^+$ regulatory T cells (4, 5). Although it appears very likely that most $CD4^+$ $CD25^+$ regulatory T cell development occurs in the thymus, accumulating evidence suggests that these cells may also develop in the periphery (i.e., extrathymically). For example, although under normal circumstances all $CD4^+$ cells from $RAG^{-/-}$ TCR transgenic mice are $CD25^-$, studies have shown that a percentage of $RAG^{-/-}$ TCR transgenic T cells adoptively transferred into antigen-expressing transgenic mice or mice that have received a tolerizing dose of peptide antigen administered either i.v. or orally can convert to a $CD4^+$ $CD25^+$ regulatory T cell phenotype (6, 7). It is unclear, however, whether $CD4^+$ $CD25^-$ T cells can or do convert to a $CD4^+$ $CD25^+$ regulatory T cell phenotype under natural conditions, i.e., with

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expression of the natural TCR repertoire and exposure to the natural endogenous antigen load. The purpose of this study was to address this issue and identify the requirements for this conversion process. We have found that mature peripheral $CD4^+$ $CD25^-$ T cells can indeed convert to a $CD4^+$ $CD25^+$ regulatory T cell phenotype and do so in a thymus-independent but B7-dependent manner.

## RESULTS

### $CD4^+$ $CD25^-$ T cells can convert into $CD4^+$ $CD25^+$ T cells in vivo in both sublethally irradiated and nonirradiated mice

It is not clear whether the $CD4^+$ $CD25^+$ T cells represent a distinct lineage of cells that develops exclusively in the thymus or whether these cells can be induced in the periphery. The following experiments were designed to determine whether $CD4^+$ $CD25^-$ T cells from wild-type mice are capable of converting to a $CD4^+$ $CD25^+$ regulatory T cell phenotype in vivo. LN and spleen cells were harvested from congenic $CD45.1^+$ mice and $CD4^+$ $CD25^-$ T cells were sorted to >99.7% purity (Fig. 1 A). $10 \times 10^6$ $CD45.1^+$ $CD4^+$ $CD25^-$ T cells were injected i.v. into sublethally irradiated $CD45.2^+$ mice. Blood was collected weekly and LN, spleen, and thymus were harvested either 1 or 6 wk after injection of cells. Donor $CD45.1^+$ $CD4^+$ cells were gated (Fig. 1 B, left) and analyzed for the presence of $CD45.1^+$ $CD4^+$ $CD25^+$ T cells. The $CD45.1^+$ $CD4^+$ $CD25^+$ T cells (2–4%) could be detected in blood (not depicted), LN (Fig. 1 B, middle), and spleen (see Fig. S1, middle, which is available at http://www.jem.org/cgi/content/full/jem.20041201/DC1), but not thymus (not depicted), as early as 1 wk after i.v. injection of $CD45.1^+$ $CD4^+$ $CD25^-$ T cells. By 6 wk after injection, 5–12% of the transferred $CD45.1^+$ cells expressed CD25 in LN (Fig. 1 B, right) as well as in the thymus (not depicted). The percentage of $CD25^+$ cells in the spleen was somewhat less and averaged ~5–7% (Fig. S1, right).

Sublethal levels of irradiation create significant "space" in the immune compartment and induce T cells to undergo dramatic homeostatic proliferation to fill this space. There is, therefore, a possibility that the $CD4^+$ $CD25^-$ cells introduced to this environment may up-regulate CD25 as a consequence

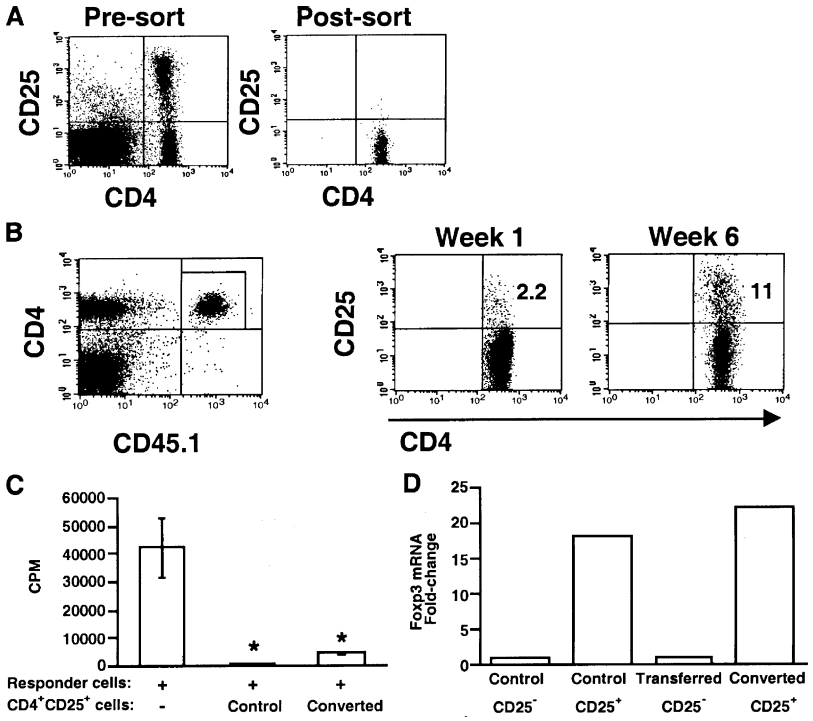


**Figure 1. $CD4^+$ $CD25^-$ cells convert into $CD4^+$ $CD25^+$ cells that exhibit regulatory function in sublethally irradiated mice.** (A) Purity of $CD4^+$ $CD25^-$ cells for transfer. $CD4^+$ $CD25^-$ cells from LN and spleen cells of $CD45.1^+$ mice were purified using T and CD4 cell affinity columns, magnetic beads, and high speed sorting. $CD4^+$ $CD25^+$ cells account for ~10% of $CD4^+$ cells before sorting (left). $CD4^+$ $CD25^-$ cells were purified to >99.7% (<0.3% contamination with $CD4^+$ $CD25^+$ cells; right). (B) $10 \times 10^6$ million purified $CD45.1^+$ $CD4^+$ $CD25^-$ cells were transferred into sublethally irradiated $CD45.2^+$ recipients. (B) LN cells or (C and D) LN and spleen cells were collected and labeled for CD45.1, CD4, and CD25. $CD45.1^+$ $CD4^+$ cells (B) were gated and analyzed for the presence of converted $CD4^+$ $CD25^+$ cells 1 (middle) and 6 wk (right) after transfer or 6 wk after transfer and were (C) tested for regulatory function or (D) Foxp3 expression. (C) To test for regulatory function, 25,000 freshly harvested $CD4^+$ $CD25^-$ cells (Responder cells) were purified and cocultured at a 1:1 (regulatory/responder) ratio with either freshly harvested $CD4^+$ $CD25^+$ cells (Control) or sorted $CD45.1^+$ $CD4^+$ $CD25^+$ cells (Converted) in the presence of irradiated spleen cells (APCs) and anti-CD3 for 3 d. (D) For quantitative analysis of Foxp3 expression, freshly harvested $CD4^+$ $CD25^-$ (Control) or $CD4^+$ $CD25^+$ (Control) cells, or $CD45.1^+$ $CD4^+$ $CD25^-$ (Transferred) or $CD45.1^+$ $CD4^+$ $CD25^+$ (Converted) cells were sorted and Foxp3 mRNA levels were quantified by real-time PCR. Data are presented as normalized Foxp3 mRNA expression levels in the samples relative to normalized Foxp3 mRNA expression levels in the control $CD4^+$ $CD25^-$ cells (fold change = 1). *, a significant difference from the positive control ($P < 0.01$).

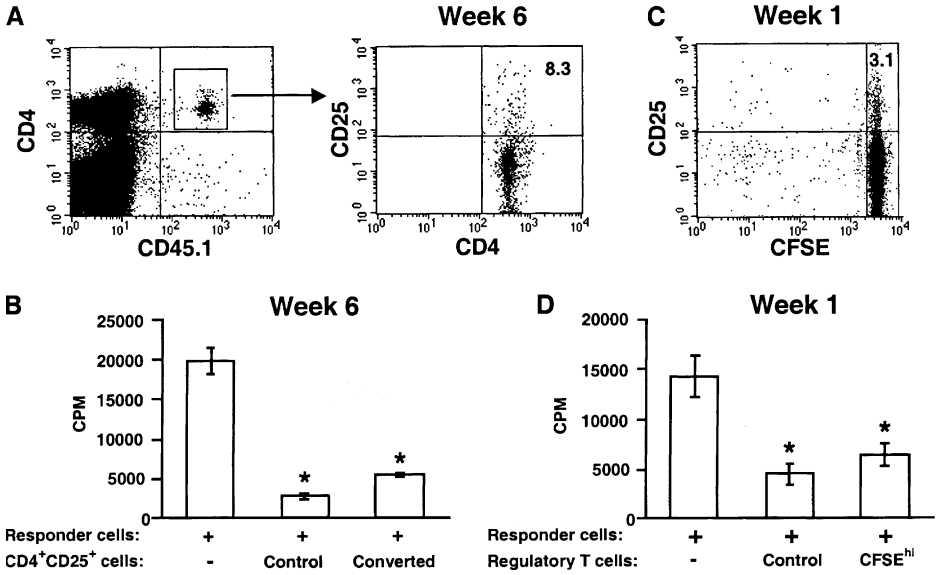


**Figure 2. CD4$^+$ CD25$^-$ cells convert into CD4$^+$ CD25$^+$ cells that exhibit regulatory function in nonirradiated mice.** $10 \times 10^6$ purified CD45.1$^+$ CD4$^+$ CD25$^-$ cells were transferred into nonirradiated CD45.2$^+$ recipients and lymphoid cells collected 6 wk after transfer. LN cells were labeled for CD45.1, CD4, and CD25, and (A) CD45.1$^+$ CD4$^+$ cells were gated and then analyzed for the presence of converted CD4$^+$ CD25$^+$ cells or (B) CD45.1$^+$ CD4$^+$ CD25$^+$ cells were sorted and tested for regulatory function. $10 \times 10^6$ purified CD45.1$^+$ CD4$^+$ CD25$^-$ cells were also labeled with CFSE and then transferred into nonirradiated CD45.2$^+$ recipients. 1 wk after transfer, lymphoid cells were collected and (C) CD45.1$^+$ CD4$^+$ cells were analyzed for the presence of CFSE$^{hi}$ CD25$^+$ cells or (D) pooled and CFSE$^{hi}$ CD45.1$^+$ CD4$^+$ CD25$^+$ cells sorted by high speed sorter and tested for regulatory function in vitro. To test for regulatory function, 12,000 freshly harvested CD4$^+$ CD25$^-$ cells (Responder cells) were purified and cocultured at a 1:4 (regulatory/responder) ratio with either freshly harvested CD4$^+$ CD25$^+$ cells (Control) or (B) CD45.1$^+$ CD4$^+$ CD25$^+$ cells (Converted), or (D) CFSE$^{hi}$ CD45.1$^+$ CD4$^+$ CD25$^+$ cells (CFSE$^{hi}$) in the presence of irradiated spleen cells (APC) and anti-CD3 for 3 d. *, a significant difference from the positive control ($P < 0.01$).

of this homeostatic proliferation. To determine whether the conversion of CD4$^+$ CD25$^-$ cells into CD4$^+$ CD25$^+$ cells can occur in the absence of the homeostatic proliferation found in the sublethally irradiated (lymphopenic) mice, CD45.1$^+$ CD4$^+$ CD25$^-$ T cells were injected into normal nonirradiated CD45.2$^+$ recipients. The percentage (and numbers) of total CD45.1$^+$ CD4$^+$ cells recovered from all of the lymphoid organs 6 wk later was extremely low by comparison to sublethally irradiated animals (sublethally irradiated: $\geq$12% vs. nonirradiated: $\leq$0.5%). Despite the scarcity of the transferred CD45.1$^+$ cells, the relative percentage of CD4$^+$ CD25$^+$ cells found in nonirradiated mice was similar to that found in sublethally irradiated mice. In nonirradiated mice, 7–12% of transferred cells expressed CD25 in the LN (Fig. 2 A, right), and 4–7% of transferred cells expressed CD25 in the spleen (not depicted). Transferred CD45.1$^+$ CD4$^+$ cells were virtually undetectable in the thymus of these mice. Based on these results, it appears that CD4$^+$ CD25$^-$ cells can convert to a CD25$^+$ phenotype whether the cells are transferred into a lymphopenic environment and subsequently undergo homeostatic proliferation, or whether they are transferred into a "normal" environment where they undergo very limited homeostatic proliferation.

### Converted CD4$^+$ CD25$^+$, but not transferred CD4$^+$ CD25$^-$, T cells exhibit characteristics of the naturally occurring CD4$^+$ CD25$^+$ regulatory T cells

Because CD25, the IL-2R$\alpha$ chain, is an activation marker for T cells as well as a marker for a population of naturally occurring regulatory T cells (2), the up-regulation of CD25 on a small percentage of initially CD4$^+$ CD25$^-$ cells could conceivably be the result of activation of these cells after injection. The CD4$^+$ CD25$^+$ regulatory T cells are characterized by their failure to proliferate in response to stimulation in vitro (anergy), their ability to suppress responder T cell (CD4$^+$ CD25$^-$ T cell) proliferation in vitro, high levels of Foxp3 expression, and expression of an array of typical surface markers (8–17). The following series of experiments were designed to determine whether the converted CD4$^+$ CD25$^+$ T cells exhibit the same characteristics as naturally occurring CD4$^+$ CD25$^+$ regulatory T cells. For these experiments, CD45.1$^+$ CD4$^+$ CD25$^-$ T cells were injected into either sublethally irradiated or nonirradiated CD45.2$^+$ mice. After 6 wk, LNs and spleens were harvested from CD45.2$^+$ mice that had received CD45.1$^+$ CD4$^+$ CD25$^-$ T cells, and the converted CD45.1$^+$ CD4$^+$ CD25$^+$ cells and CD45.1$^+$ CD4$^+$ CD25$^-$ cells were sorted. For the functional assays, converted CD45.1$^+$ CD4$^+$ CD25$^+$ cells stimulated in vitro in the presence of irradiated spleen cells as APCs and anti-CD3 did not proliferate; i.e., these cells were anergic (Fig. S2 A, available at http://www.jem.org/cgi/content/full/jem.20041201/DC1), whereas transferred CD45.1$^+$ CD4$^+$ CD25$^-$ T cells that did not convert to a CD25$^+$ phenotype proliferated readily in response to stimulation in vitro (Fig. S2 B). In a standard assay for the analysis of in vitro regulatory activity, converted CD45.1$^+$ CD4$^+$ CD25$^+$ T cells from both sublethally irradiated and nonirradiated mice T cells suppressed proliferation of

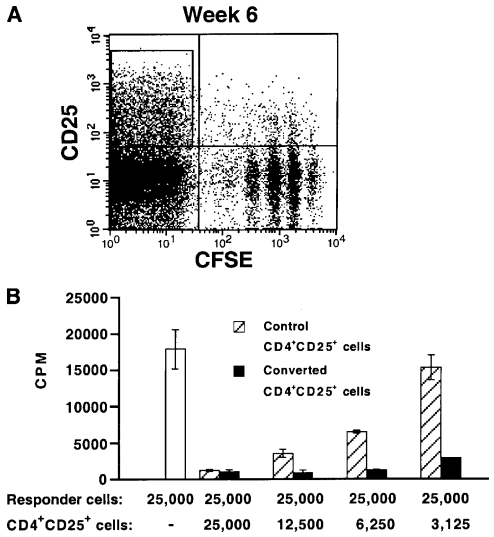


**Figure 3. Converted CD4$^+$ CD25$^+$ cells that undergo homeostatic proliferation exhibit more potent regulatory function in vitro than freshly harvested CD4$^+$ CD25$^+$ cells.** $10 \times 10^6$ purified CD45.1$^+$ CD4$^+$ CD25$^-$ cells were labeled with CFSE and then transferred into sublethally irradiated CD45.2$^+$ recipients. 6 wk after transfer, LN and spleen cells were collected, pooled, and (A) CFSE$^{lo}$ CD45.1$^+$ CD4$^+$ CD25$^+$ cells were sorted by high speed sorter and (B) tested for regulatory function in vitro. For this assay, 25,000 freshly harvested CD4$^+$ CD25$^-$ cells (Responder cells) were purified and cocultured at varying (regulatory/responder) ratios with either freshly harvested CD4$^+$ CD25$^+$ cells (Control) or CFSE$^{lo}$ CD45.1$^+$ CD4$^+$ CD25$^+$ cells (Converted) in the presence of irradiated spleen cells (APC) and anti-CD3 for 3 d.

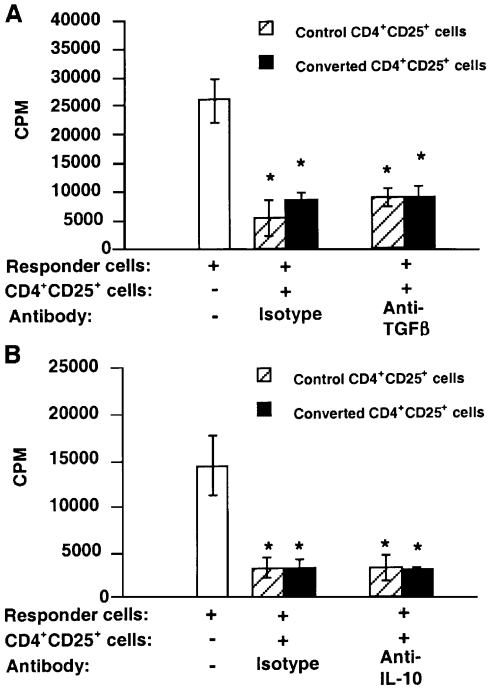


**Figure 4. Converted CD4$^+$ CD25$^+$ cells do not suppress responder cell proliferation through the production of TGFβ or IL-10.** $10 \times 10^6$ purified CD45.1$^+$ CD4$^+$ CD25$^-$ cells were transferred into nonirradiated CD45.2$^+$ recipients. 6 wk after transfer, lymphoid cells were collected, pooled, and CD45.1$^+$ CD4$^+$ CD25$^+$ cells were sorted by high speed sorter and tested for regulatory function in vitro. For this assay, 12,000 freshly harvested CD4$^+$ CD25$^-$ cells (Responder cells) were purified and cocultured at a 1:4 (regulatory/responder) ratio with either freshly harvested CD4$^+$ CD25$^+$ cells (Control) or CD45.1$^+$ CD4$^+$ CD25$^+$ cells (Converted) in the presence of irradiated spleen cells (APC) and either isotype, (A) anti-TGFβ, or (B) 10 μg/ml anti–IL-10 antibodies and anti-CD3 for 3 d. *, a significant difference from the positive control ($P < 0.01$).

responder cells (Figs. 1 C and 2 B, respectively), whereas CD45.1$^+$ CD4$^+$ CD25$^-$ T cells did not (not depicted).

Naturally occurring CD4$^+$ CD25$^+$ regulatory cells that have undergone homeostatic proliferation in vivo exhibit more potent regulatory function in in vitro assays (18). To determine whether transferred cells that have undergone homeostatic proliferation can convert and exhibit more potent regulatory function, purified CD45.1$^+$ CD4$^+$ CD25$^-$ cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and transferred into sublethally irradiated CD45.2$^+$ recipients. 6 wk later, converted CFSE$^{lo}$ CD45.1$^+$ CD4$^+$ CD25$^+$ cells (i.e., cells that had undergone homeostatic division; Fig. 3 A) were sorted and tested for their ability to suppress at various regulatory/responder cell ratios. Converted CD45.1$^+$ CD4$^+$ CD25$^+$ cells that had undergone homeostatic proliferation were much more effective at suppressing responder proliferative responses at lower regulatory/responder cell ratios than freshly harvested control CD4$^+$ CD25$^+$ regulatory T cells, indicating that these converted cells possess greater regulatory capacity (Fig. 3 B). In the next experiment, converted CD4$^+$ CD25$^+$ T cells were tested for the possibility that they mediate suppression in vitro through the production of the immunosuppressive cytokines, TGFβ and IL-10 (19–22). Similar to what some investigators have reported for naturally occurring CD4$^+$ CD25$^+$ regulatory T cells, converted CD4$^+$ CD25$^+$ T cells from either nonirradiated (Fig. 4, A and B) or sublethally irradiated (not depicted) mice do not suppress responder cell proliferation in vitro through the production of either TGFβ or IL-10 (Fig. 4, A and B, respectively). This was found using either 10 or 50 μg/ml of antibody (not depicted).

In the next test for the CD4$^+$ CD25$^+$ regulatory T cell phenotype, expression of the transcription factor, Foxp3, was analyzed by real-time PCR in sorted converted CD45.1$^+$ CD4$^+$ CD25$^+$ T cells and in transferred CD45.1$^+$ CD4$^+$ CD25$^-$ T cells that did not convert to a CD25$^+$ phenotype. Converted CD45.1$^+$ CD4$^+$ CD25$^+$, but not CD45.1$^+$ CD4$^+$ CD25$^-$, T cells expressed high levels of Foxp3 mRNA that were comparable to control CD4$^+$ CD25$^+$ regulatory T cells (Fig. 1 D).

The final experiment in this series examined whether converted CD4$^+$ CD25$^+$ cells express the typical surface markers that are associated with naturally occurring CD4$^+$ CD25$^+$ regulatory cells. Although glucocorticoid-induced TNF receptor gene (GITR) and cytotoxic T lymphocyte–associated antigen 4 (CTLA-4) are also expressed by activated T cells, they are expressed at constitutively high levels by naturally occurring CD4$^+$ CD25$^+$ regulatory T cells and appear to be involved at some level in the function of these cells

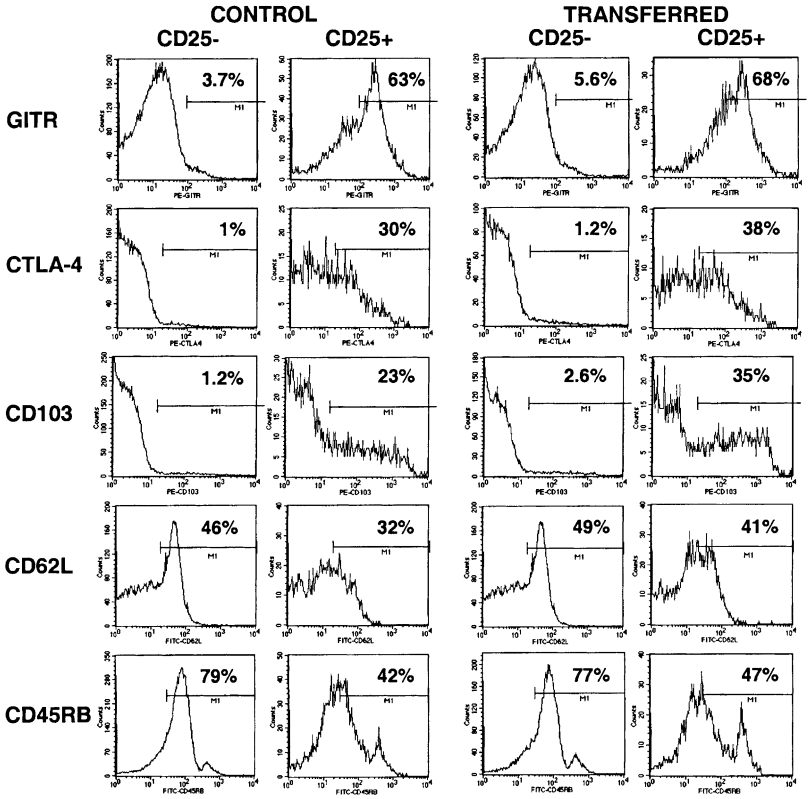


**Figure 5. Expression of surface markers GITR, CTLA-4, CD103, CD62L, and CD45RB is similar between converted and freshly harvested CD4$^+$ CD25$^+$ cells.** $10 \times 10^6$ purified CD45.1$^+$ CD4$^+$ CD25$^-$ cells were transferred into sublethally irradiated CD45.2$^+$ recipients. 6 wk after transfer, lymphoid cells were collected and labeled for CD45.1, CD4, CD25, and either GITR, CTLA-4, CD103, CD62L, or CD45RB. Freshly harvested or converted CD45.1$^+$ CD4$^+$ CD25$^+$ or CD25$^-$ cells were gated and analyzed for expression of surface markers.

(13–15, 17). Expression of high levels of either CD62L or CD103 is associated with more potent suppressive function in vitro (11, 16). CD45RB$^{lo}$ expression identifies another population of regulatory T cells that controls colitis and may overlap in function with the CD4$^+$ CD25$^+$ regulatory cells (12). In the following experiments, expression of these markers was compared between converted CD45.1$^+$ CD4$^+$ CD25$^+$ cells and naturally occurring CD4$^+$ CD25$^+$ regulatory T cells. In general, converted CD4$^+$ CD25$^+$ T cells express all of the markers that are typically expressed by naturally occurring CD4$^+$ CD25$^+$ regulatory T cells (Fig. 5). The profiles were similar between the converted and naturally occurring CD4$^+$ CD25$^+$ T cells. There were also no differences in surface marker profiles between transferred and freshly harvested CD4$^+$ CD25$^-$ T cells (Fig. 5).

Taken together, these data strongly suggest that a percentage of CD4$^+$ CD25$^-$ T cells can be converted into CD4$^+$ CD25$^+$ T cells that display function and phenotype that are virtually indistinguishable from that of naturally occurring CD4$^+$ CD25$^+$ regulatory T cells.

### Conversion of CD4$^+$ CD25$^-$ cells into CD4$^+$ CD25$^+$ regulatory cells is not the result of expansion of residual CD4$^+$ CD25$^+$ cells or selective CD4$^+$ CD25$^+$ cell survival

We have interpreted the appearance of CD4$^+$ CD25$^+$ cells after transfer of CD4$^+$ CD25$^-$ cells to indicate that conversion had occurred. However, an alternative possibility is that the tiny, almost undetectable (<0.3% by FACS) population of CD25$^+$ cells transferred along with the CD4$^+$ CD25$^-$ cells could have expanded to give the appearance that the CD4$^+$ CD25$^-$ cells had converted into CD4$^+$ CD25$^+$ cells. To address this issue, sorted CD45.1$^+$ CD4$^+$ CD25$^-$ T cells were labeled with CFSE and then injected into nonirradiated CD45.2$^+$ mice to avoid homeostatic proliferation. After 1 wk, CD45.1$^+$ CD4$^+$ CD25$^+$ cells that were CFSE$^{hi}$ (Fig. 2 C) and had not undergone proliferation were sorted and tested for their ability to suppress in vitro. Although only 2–4% of the cells expressed CD25 (Fig. 2 C), these cells were able to suppress responder cell proliferation in vitro (Fig. 2 D). To further address this issue, absolute numbers of recovered cells (CD45.1$^+$) were analyzed 6 wk after transfer into nonirradiated or sublethally irradiated mice. The purpose of the first analysis was to determine the absolute number of CD4$^+$ CD25$^+$ cells that could be recovered when known numbers of CD4$^+$ CD25$^+$ cells were injected. $10^6$, 100,000, or 30,000 purified conventional CD45.1$^+$ CD4$^+$ CD25$^+$ cells were injected into nonirradiated mice (CD45.2$^+$), and then transferred cells were analyzed 6 wk later. CD45.1$^+$ CD4$^+$ CD25$^+$ cells were barely detectable ($\leq$1,000) in mice that had received either 30,000 or 100,000 conventional CD45.1$^+$ CD4$^+$ CD25$^+$ cells, whereas 5,742 $\pm$ 1,969 CD45.1$^+$ CD4$^+$ CD25$^+$ cells (Table S1, available at http://www.jem.org/cgi/content/full/

jem.20041201/DC1) could be detected in mice that had received $10^6$ conventional CD45.1$^+$ CD4$^+$ CD25$^+$ cells. The transferred CD4$^+$ CD25$^-$ cells could potentially contain 0.3% contamination (refer to Fig. 1 A) of a trace population of CD4$^+$ CD25$^+$ cells, which would equate to a total number of ~30,000 of the $10 \times 10^6$ CD4$^+$ CD25$^-$ cells injected. However, as many as 15,776 ± 1,797 CD4$^+$ CD25$^+$ cells (Table S1) could be recovered 6 wk after transfer of CD4$^+$ CD25$^-$ cells, which, if we assume that the transferred CD4$^+$ CD25$^-$ cell population consistently contains 30,000 CD4$^+$ CD25$^+$ cells, would correspond to a >50% recovery of this trace population. This seems highly unlikely considering ≤1,000 or only 3% of the 30,000 conventional CD4$^+$ CD25$^+$ cells that were intentionally injected could be recovered 6 wk after transfer. Similarly, in sublethally irradiated mice that received $10^6$ conventional CD45.1$^+$ CD4$^+$ CD25$^+$ cells (i.e., >30 times the 30,000 trace CD4$^+$ CD25$^+$ population contaminating the transferred CD4$^+$ CD25$^-$ cells), only 169,779 ± 31,419 CD4$^+$ CD25$^+$ cells (i.e., 17% of the starting population or representing a fivefold decrease) were recovered after 6 wk. On the other hand, 442,173 ± 49,106 CD4$^+$ CD25$^+$ cells (Table S1) were recovered from mice that had received $10 \times 10^6$ CD4$^+$ CD25$^-$ cells 6 wk earlier, which would actually equate to a 13-fold increase in the 30,000 CD4$^+$ CD25$^+$ cells contaminating the transferred CD4$^+$ CD25$^-$ population. Based on the numbers described above, it would be impossible for the progeny of a trace population of 30,000 CD4$^+$ CD25$^+$ cells to expand to the numbers found 6 wk after transfer of CD45.1$^+$ CD4$^+$ CD25$^-$ cells into either nonirradiated or sublethally irradiated mice. The data presented in Table S1 also show that CD4$^+$ CD25$^+$ cells (0.6% recovery in nonirradiated and 17% recovery in irradiated mice) do not exhibit increased survival over the CD4$^+$ CD25$^-$ cells (3.3% recovery in nonirradiated and 80% recovery in irradiated mice) in these systems. Therefore, these data also rule out selective survival of the CD4$^+$ CD25$^+$ cells over the CD4$^+$ CD25$^-$ cells as a possible explanation for the presence of these cells. In summary, the numerical data described above strongly suggest that the appearance of CD4$^+$ CD25$^+$ cells after transfer of CD4$^+$ CD25$^-$ cells was not the result of expansion of a small contaminating population of CD25$^+$ cells or selective survival of this trace population, but could only be attributed to the actual conversion of the CD4$^+$ CD25$^-$ T cells into CD4$^+$ CD25$^+$ regulatory T cells.

### Conversion of CD4$^+$ CD25$^-$ T cells into functional CD4$^+$ CD25$^+$ regulatory T cells does not require the thymus

The thymus is generally thought to be the site of CD4$^+$ CD25$^+$ regulatory T cell development, and small numbers of mature peripheral T cells can apparently recirculate back through the thymus (23). There is, therefore, a possibility that conversion of CD4$^+$ CD25$^-$ T cells to CD4$^+$ CD25$^+$ regulatory T cells may occur in the thymus. The following experiments were designed to test this possibility. Adult CD45.2$^+$ mice were thymectomized, and 4 wk later, sorted CD45.1$^+$ CD4$^+$ CD25$^-$ cells were injected into sublethally or nonirradiated thymectomized mice. Blood was collected weekly, and LN and spleen were harvested at 6 wk after injection and analyzed for the presence of CD45.1$^+$ CD4$^+$ CD25$^+$ T cells. CD45.1$^+$ CD4$^+$ CD25$^+$ T cells could be detected in blood as early as 1–2 wk after injection (not depicted). In the sublethally irradiated thymectomized mice, 6–14% of the CD45.1$^+$ CD4$^+$ cells expressed CD25 in the LN (Fig. 6 A) and 2–5% expressed CD25 in the spleen (not depicted) 6 wk after transfer. Similar data were found in nonirradiated thymectomized mice. Very few total CD45.1$^+$ CD4$^+$ cells (sublethally irradiated: ≥25% vs. nonirradiated: ≤1.5%) were recovered (similar to that found in nonirradiated intact mice, see above), and of the CD45.1$^+$ cells that were recovered, 7–11% of the CD45.1$^+$ CD4$^+$ cells in the LN and 3–4% of the CD45.1$^+$ CD4$^+$ cells in the spleen expressed CD25 (not depicted). These data indicate that CD4$^+$ CD25$^-$ cells can at least up-regulate CD25 in the absence of the thymus in both sublethally and nonirradiated mice.

The next series of experiments was designed to determine whether these cells that have converted to a CD25$^+$ phenotype in the absence of the thymus gland exhibit regulatory function characteristic of naturally occurring CD4$^+$ CD25$^+$ regulatory T cells. CD45.1$^+$ CD4$^+$ CD25$^+$ cells, but not the unconverted CD45.1$^+$ CD4$^+$ CD25$^-$ cells (not depicted), harvested from sublethally irradiated thymectomized mice were able to suppress the proliferation of responder cells to the same extent as the control CD4$^+$ CD25$^+$ regulatory T cells (Fig. 6 B). Furthermore, CD45.1$^+$ CD4$^+$ CD25$^+$ cells, but not CD45.1$^+$ CD4$^+$ CD25$^-$ cells, harvested from nonirradi-

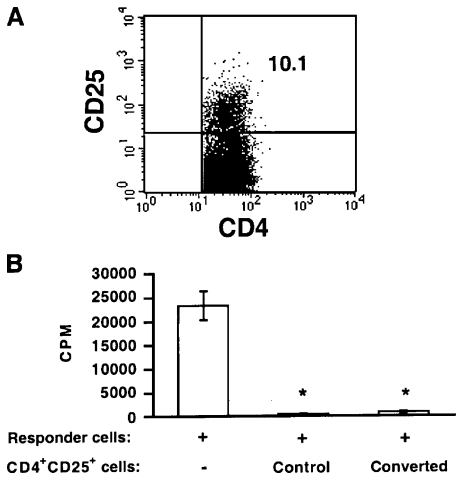


**Figure 6. CD4$^+$ CD25$^+$ cells can be converted in the absence of the thymus.** $10 \times 10^6$ purified CD45.1$^+$ CD4$^+$ CD25$^-$ cells were transferred into thymectomized sublethally irradiated CD45.2$^+$ recipients. 6 wk after transfer, lymphoid cells were collected and either (A) labeled for CD45.1, CD4, and CD25, with CD45.1$^+$ CD4$^+$ cells gated and analyzed for the presence of converted CD4$^+$ CD25$^+$ cells, or (B) pooled and CD45.1$^+$ CD4$^+$ CD25$^+$ sorted by high speed sorter and tested for regulatory function in vitro. For this assay, 25,000 freshly harvested CD4$^+$ CD25$^-$ cells (Responder cells) were purified and cocultured at a 1:1 (regulatory/responder) ratio with either freshly harvested CD4$^+$ CD25$^+$ cells (Control) or (B) CD45.1$^+$ CD4$^+$ CD25$^+$ (Converted) cells in the presence of irradiated spleen cells (APC) and anti-CD3 for 3 d. *, a significant difference from the positive control ($P < 0.01$).

ated thymectomized mice were also able to suppress the proliferation of responder cells to the same extent as the control $CD4^+$ $CD25^+$ regulatory T cells (not depicted). In the final test for characteristics typical of $CD4^+$ $CD25^+$ regulatory T cells, $CD45.1^+$ $CD4^+$ $CD25^+$ cells from sublethally irradiated thymectomized mice were analyzed for expression of Foxp3. Converted $CD45.1^+$ $CD4^+$ $CD25^+$ T cells, but not $CD45.1^+$ $CD4^+$ $CD25^-$ cells, from thymectomized mice expressed high levels of Foxp3 mRNA that were comparable to control $CD4^+$ $CD25^+$ regulatory T cells (not depicted). Taken together, these data indicate that the conversion of $CD4^+$ $CD25^-$ T cells into functional $CD4^+$ $CD25^+$ regulatory T cells occurs in the periphery in the absence of the thymus.

### Conversion of $CD4^+$ $CD25^-$ T cells into functional $CD4^+$ $CD25^+$ regulatory T cells requires costimulation via B7.1/B7.2

Several reports have suggested that signaling through CD28 and/or CTLA-4 via B7.1/B7.2 is required for the development and maintenance of $CD4^+$ $CD25^+$ regulatory T cells (24–26). The following experiments were designed to determine whether costimulation via B7.1/B7.2 is required for conversion of $CD4^+$ $CD25^-$ T cells into functional $CD4^+$ $CD25^+$ regulatory T cells. For these experiments, $CD45.1^+$ $CD4^+$ $CD25^-$ cells were injected into $CD45.2^+$ B7.1/B7.2$^{-/-}$ mice. LN and spleen were harvested 6 wk after injection and analyzed for the presence of converted $CD45.1^+$ $CD4^+$ $CD25^+$ T cells. Very few $CD45.1^+$ $CD4^+$ cells were recovered, and only 2–4% of the injected $CD45.1^+$ $CD4^+$ T cells expressed CD25 compared with the 7–12% found in B6 mice (Fig. 7 A, top, and B, left). Converted $CD45.1^+$ $CD4^+$ $CD25^+$ T cells were sorted and tested for their ability to suppress proliferation of responder cells in vitro. Sorted $CD45.1^+$ $CD4^+$ $CD25^+$ T cells from B7.1/B7.2$^{-/-}$ mice were unable to suppress the proliferation of responder cells 6 wk after transfer (Fig. 7 C).

Because CD28 costimulation via B7 is required for $CD4^+$ $CD25^+$ regulatory cell homeostasis in the periphery (24–26), it was necessary to rule out the possibility that the $CD4^+$ $CD25^-$ cells could have converted to $CD4^+$ $CD25^+$ cells initially (i.e., within the first week after transfer as found in wild-type mice), but then failed to survive until collection at 6 wk after transfer. Previous studies have found that $CD4^+$ $CD25^+$ cells are dramatically decreased after 2 wk in the absence of B7 costimulation (26). To determine whether $CD4^+$ $CD25^+$ cells can survive for at least 1 wk in the absence of B7 costimulation, $10^6$ purified conventional $CD45.1^+$ $CD4^+$ $CD25^+$ cells were transferred into either wild-type B6 or B7.1/B7.2$^{-/-}$ mice. 1 wk later, absolute numbers of $CD45.1^+$ $CD4^+$ $CD25^+$ cells were determined. There were no significant differences between the absolute numbers of $CD45.1^+$ $CD4^+$ $CD25^+$ cells recovered from B6 (40,106 ± 1,730) and B7.1/ B7.2$^{-/-}$ (47,450 ± 9,879)

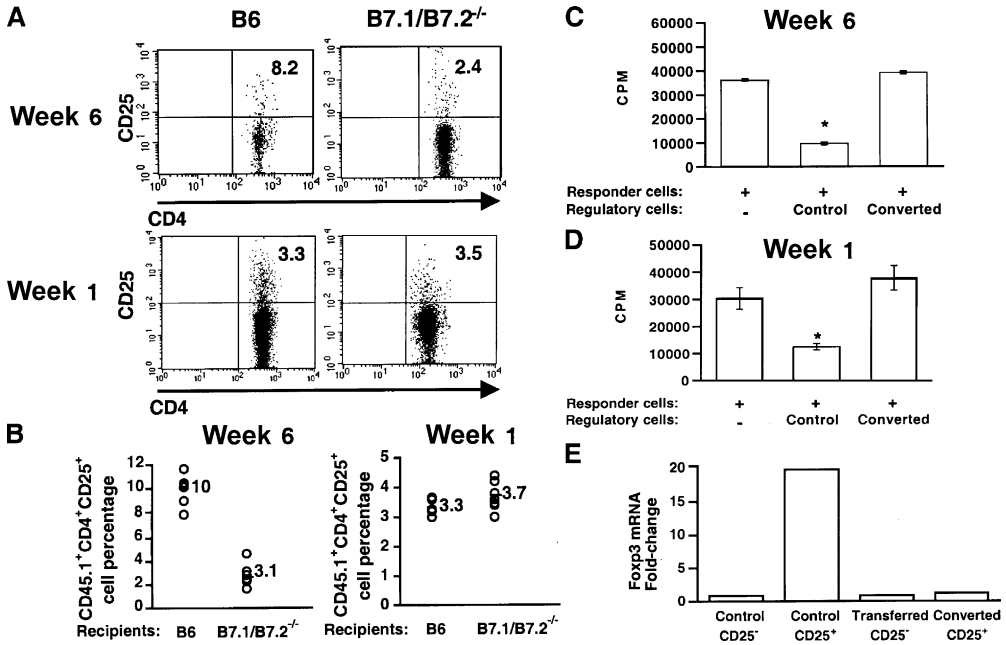


**Figure 7. $CD4^+$ $CD25^+$ cells cannot be converted in the absence of B7 costimulation.** $10 \times 10^6$ purified $CD45.1^+$ $CD4^+$ $CD25^-$ cells were transferred into nonirradiated wild-type (B6) or B7.1/B7.2$^{-/-}$ ($CD45.2^+$) recipients. (A and B) 6 or 1 wk after transfer, LN cells were collected and labeled for CD45.1, CD4, and CD25, and $CD45.1^+$ $CD4^+$ cells were gated and analyzed by FACS for the presence of converted $CD4^+$ $CD25^+$ cells. (A) Figure represents typical dot-plot and B shows the percentage of $CD45.1^+$ $CD4^+$ $CD25^+$ cells in individual mice. LN and spleen cells were pooled and sorted for $CD45.1^+$ $CD4^+$ $CD25^+$ cells (C) 6 or (D) 1 wk after transfer into B7.1/B7.2$^{-/-}$ mice by high speed sorter and tested for regulatory function in vitro or (E) Foxp3 expression by real-time PCR (see legend to Fig. 1 for details). For the regulatory function assay, 12,000 freshly harvested $CD4^+$ $CD25^-$ cells (Responder cells) were purified and cocultured at a 1:2 (regulatory/responder) ratio with either freshly harvested $CD4^+$ $CD25^+$ cells (Control) or $CD45.1^+$ $CD4^+$ $CD25^+$ (Converted) cells in the presence of irradiated spleen cells (APC) and anti-CD3 for 3 d. *, a significant difference from the positive control ($P < 0.01$).

mice, suggesting that $CD4^+$ $CD25^+$ cells can survive comparably for at least 1 wk in the presence or absence of B7 costimulation. To confirm that $CD4^+$ $CD25^-$ cells fail to convert in the absence of B7 costimulation, $CD45.1^+$ $CD4^+$ $CD25^-$ cells were transferred into B6 or $B7.1/B7.2^{-/-}$ mice and $CD45.1^+$ $CD4^+$ $CD25^+$ cells were analyzed or sorted 1 wk later. As described above for 6 wk, 2–4% of the transferred $CD45.1^+$ cells expressed CD25 (Fig. 7 A, bottom, and B, right). However, as also described for 6 wk, these converted $CD25^+$ cells were unable to suppress proliferation (Fig. 7 D). Furthermore, these cells did not express Foxp3 mRNA (Fig. 7 E) or high levels of either GITR or CTLA-4 (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20041201/DC1), the markers characteristic of conventional $CD4^+$ $CD25^+$ regulatory cells. Because $CD4^+$ $CD25^+$ cells can survive for at least 1 wk in the absence of B7 costimulation, these results strongly suggested that the failure to find converted $CD4^+$ $CD25^+$ regulatory cells after transfer into $B7.1/B7.2^{-/-}$ mice is because these cells fail to convert in the absence of B7 costimulation. In the aggregate, these results indicate that B7 costimulation is an absolute requirement for conversion of $CD4^+$ $CD25^-$ cells into $CD4^+$ $CD25^+$ regulatory cells.

## DISCUSSION

Regulation by $CD4^+$ $CD25^+$ T cells is an important component of peripheral tolerance that is responsible for controlling autoreactive T cells in normal individuals. Where these cells originate and how they are maintained in the periphery are unknown. We show here for the first time that peripheral $CD4^+$ $CD25^-$ T cells can convert in vivo into a $CD4^+$ $CD25^+$ regulatory T cell phenotype spontaneously in a natural environment. The converted cells appear to be functionally and phenotypically indistinguishable from naturally occurring $CD4^+$ $CD25^+$ regulatory T cells. The converted cells are anergic, suppress the proliferation of $CD4^+$ $CD25^-$ responder T cells in vitro (although not through the production of TGFβ or IL-10), and express high levels of Foxp3 mRNA as well as a phenotypic profile of surface markers including GITR, CTLA-4, and CD103 at levels that are comparable to that found in naturally occurring $CD4^+$ $CD25^+$ regulatory T cells.

Previous studies using various model systems have found that $CD4^+$ $CD25^-$ cells can be induced to express CD25 and exhibit regulatory properties both in vivo and in vitro. The in vivo studies, however, used model systems consisting of TCR transgenic mice that express high affinity clonotypic TCR and supraphysiological amounts of specific nonself peptide either administered exogenously or expressed endogenously as a transgene (6, 7). Thorstenson and Khoruts (17) have shown that a percentage of OVA-specific TCR transgenic $CD4^+$ $CD25^-$ cells from $RAG^{-/-}$ mice adoptively transferred into normal mice could be induced to express CD25 and develop regulatory function after either i.v. or oral administration of a tolerizing dose of purified OVA peptide (7). Along the same lines, Apostolou et al. (6) found that a small percentage of $CD4^+$ $CD25^-$ cells also converted to a $CD25^+$ phenotype after transfer of influenza hemaglutinin-specific TCR transgenic $CD4^+$ $CD25^-$ cells on a $RAG^{-/-}$ background into transgenic mice that expressed the hemaglutinin peptide under the control of the immunoglobulin κ promoter. However, in the latter model, both the converted $CD25^+$ and the transferred $CD25^-$ cells exhibited regulatory properties, suggesting that under these conditions strong TCR signaling and/or aberrant expression of antigen by atypical APCs may induce cells that are anergic and suppressive, but does not necessarily reflect what occurs under normal conditions, i.e., with T cells expressing a normal TCR repertoire and presentation of small amounts of self-peptide. Thusly, although both of these studies provided data that suggested that $CD4^+$ $CD25^-$ cells could convert to a $CD4^+$ $CD25^+$ regulatory T cell phenotype, conversion occurred under somewhat extraordinary conditions. These studies did not address the issue of whether a naturally occurring population of $CD4^+$ $CD25^-$ cells exists that under natural conditions has the same potential to convert to a $CD4^+$ $CD25^+$ regulatory T cell phenotype. Recent studies have found that both murine and human $CD4^+$ $CD25^-$ cells can be induced in vitro to develop into $CD4^+$ $CD25^+$ T cells that exhibit many of the characteristics found in $CD4^+$ $CD25^+$ regulatory T cells including anergy, suppression of responder cell proliferation, expression of Foxp3, and in the case of murine cells, the ability to prevent allergen-induced asthma (27, 28). Taken together, these data suggest that at least some types of $CD4^+$ $CD25^-$ cells have the inherent ability to convert to a $CD4^+$ $CD25^+$ regulatory T cell phenotype, and indeed the results of this study confirm that this can happen spontaneously in a natural environment.

Several approaches were used in this study to rule out the possibility that the apparent conversion from $CD4^+$ $CD25^-$ cells into $CD4^+$ $CD25^+$ regulatory cells was simply the result of expansion and/or selective survival of a trace population of $CD4^+$ $CD25^+$ cells inadvertently transferred along with the $CD4^+$ $CD25^-$ cells. First, CFSE-labeled $CD45.1^+$ $CD4^+$ $CD25^-$ cells were transferred into nonirradiated $CD45.2^+$ recipients and the nonproliferating $CFSE^{hi}$ $CD45.1^+$ $CD4^+$ $CD25^+$ cells were found to exhibit regulatory function, suggesting that in the absence of expansion, conversion still occurs. Furthermore, analysis of absolute numbers of $CD45.1^+$ $CD4^+$ $CD25^+$ cells in both nonirradiated and sublethally irradiated mice that had received either $10 \times 10^6$ $CD45.1^+$ $CD4^+$ $CD25^-$ cells or $10^6$ $CD45.1^+$ $CD4^+$ $CD25^+$ cells demonstrated that it would be virtually impossible for the progeny of a trace population of 30,000 $CD4^+$ $CD25^+$ cells (i.e., 0.3% contamination) to account for the numbers of these cells found either 1 or 6 wk after transfer of $CD4^+$ $CD25^-$ cells (Table S1). With regard to selective survival of the trace population of $CD4^+$ $CD25^+$ cells, analysis of absolute numbers of either $CD4^+$ $CD25^+$ cells or $CD4^+$ $CD25^-$ cells recovered after injection of known numbers of the respective populations indicated that the $CD4^+$ $CD25^+$ cells do not typically exhibit greater survival tendencies than $CD4^+$ $CD25^-$

cells (in fact, the reverse might be true; Table S1). These data effectively ruled out the possibility that the $CD4^+$ $CD25^+$ cells found after transfer of $CD4^+$ $CD25^-$ cells are the result of expansion of a trace population of $CD4^+$ $CD25^+$ cells and/or their selective survival and support the premise that $CD4^+$ $CD25^-$ cells can convert into $CD4^+$ $CD25^+$ regulatory cells.

Lymphocytes placed in a lymphopenic environment will undergo homeostatic proliferation (29). Because a percentage of the transferred $CD4^+$ $CD25^-$ cells up-regulated CD25, which was indicative of some sort of "activation" event, we tested whether homeostatic proliferation was a requirement for conversion of $CD4^+$ $CD25^-$ cells to a $CD4^+$ $CD25^+$ regulatory T cell phenotype by injecting $CD4^+$ $CD25^-$ cells into nonirradiated recipients. Although the percentage and total number of $CD4^+$ donor cells recovered in sublethally irradiated mice were much larger than in mice that received no radiation treatment, $CD4^+$ $CD25^-$ cells could still convert to a $CD4^+$ $CD25^+$ regulatory T cell phenotype in the absence of homeostatic proliferation (i.e., in nonirradiated mice). These data suggest that although transferred $CD4^+$ $CD25^-$ T cells can, they apparently do not need to undergo homeostatic proliferation to convert to a $CD4^+$ $CD25^+$ regulatory T cell phenotype. Furthermore, we have shown that converted $CD4^+$ $CD25^+$ cells were able to suppress proliferation of responder cells at low regulatory/responder T cell ratios, whereas freshly harvested $CD4^+$ $CD25^+$ regulatory T cells could not. These data suggested that the converted $CD4^+$ $CD25^+$ cells, similar to the naturally occurring $CD4^+$ $CD25^+$ cells (18), became more potent suppressors in vitro after undergoing homeostatic proliferation in vivo, which is another indication that the converted $CD4^+$ $CD25^+$ cells are similar to naturally occurring $CD4^+$ $CD25^+$ regulatory cells.

Some, but not all, of the requirements for the conversion of $CD4^+$ $CD25^-$ T cells into a $CD4^+$ $CD25^+$ regulatory T cell phenotype are similar to the requirements for the development of naturally occurring $CD4^+$ $CD25^+$ regulatory T cells. Both CTLA-4 and CD28 are involved in the development of $CD4^+$ $CD25^+$ regulatory T cells, as this population of cells is dramatically decreased in mice deficient for either of these molecules (14, 17, 24–26). More importantly, B7 appears to be required for the peripheral maintenance of this population (24–26). Similar to the requirements for development of naturally occurring $CD4^+$ $CD25^+$ regulatory T cells, the conversion of $CD4^+$ $CD25^-$ T cells into $CD4^+$ $CD25^+$ regulatory T cells also requires costimulation via B7. On the other hand, most naturally occurring $CD4^+$ $CD25^+$ regulatory T cell development does appear to occur in the thymus (3, 30), whereas conversion of $CD4^+$ $CD25^-$ T cells to a $CD4^+$ $CD25^+$ regulatory T cell phenotype does not require the presence of the thymus. These data suggest that the mechanism of induction might be similar for both populations, whereas the site of induction may differ; the thymus might be necessary for naturally occurring $CD4^+$ $CD25^+$ regulatory T cell development, and a peripheral (i.e., an extrathymic) site appears to be sufficient for conversion of $CD4^+$ $CD25^-$ T cells. These data corroborate those found in another study, which showed that $CD4^+$ $CD25^-$ T cells can be converted to a $CD4^+$ $CD25^+$ regulatory phenotype after exposure to exogenous antigen in the periphery (31).

To date, nothing is known about the $CD4^+$ $CD25^-$ T cells that convert to a $CD4^+$ $CD25^+$ regulatory T cell phenotype. For example, it is not clear whether the $CD4^+$ $CD25^-$ cells that convert to a $CD4^+$ $CD25^+$ regulatory T cell phenotype represent a population of cells that was originally $CD25^+$ and/or whether they represent a population that arises de novo in the periphery either from a population that is predestined to become $CD4^+$ $CD25^+$ regulatory cells, or from cells that have the potential to also become effector cells. These issues warrant further study.

In conclusion, normal $CD4^+$ $CD25^-$ cells have the potential to convert spontaneously in the periphery into $CD4^+$ $CD25^+$ regulatory T cells that are physically and functionally indistinguishable from naturally occurring $CD4^+$ $CD25^+$ regulatory T cells. This conversion occurs under natural (either lymphopenic or nonlymphopenic) conditions, i.e., using a normal T cell repertoire and in the presence of the natural array of endogenous antigens. The conversion of $CD4^+$ $CD25^-$ cells into $CD4^+$ $CD25^+$ cells in a normal environment may contribute significantly to the maintenance and, therefore, homeostasis of the peripheral $CD4^+$ $CD25^+$ regulatory T cell population. A defect in this conversion process could result in a peripheral $CD4^+$ $CD25^+$ regulatory cell population of insufficient size to control the autoreactive T cells that escape negative selection and have the potential to mediate autoimmune disease.

## MATERIALS AND METHODS

**Mice.** 6–8-wk-old female and male C57BL/6 ($CD45.2^+$), B.6SJL-$Ptprc^aPep3^b$/BoyJ ($CD45.1^+$), and B7.1/B7.2 knockout mice were purchased from The Jackson Laboratory and maintained in the animal facility at the University of Louisville. Animals were maintained under the guidelines stipulated by the University of Louisville Institutional Animal Care and Use Committee.

**FACS analysis and antibodies.** The following reagents were used: FITC–, PE–, and PerCP–anti-CD4; FITC–, PE–, and biotinylated anti-CD25; FITC–anti-CD45RB, anti-CD62L, and anti-CD45.1; PE–anti-CD103, anti–CTLA-4, and anti-CD45.1; biotinylated CD45.2 antibodies and FITC-, PE-, PERCP-, and APC-labeled streptavidin; anti-CD3 antibody (BD Biosciences) and PE–anti-GITR, TGFβ, IL-10, and isotype antibodies (R&D Systems); and CFSE (Molecular Probes). For FACS analysis, cells were incubated with antibodies and staining buffer (Dulbecco's PBS, 0.1% BSA, and 0.01% $Na_2$ azide) for 15 min and then washed and analyzed on a FACScan or FACSCalibur (Becton Dickinson).

**Tracking adoptively transferred cells.** The CD45 isotype was used as a marker to track cells in the recipients. Lymphocytes were obtained from LNs and spleen and processed in HBSS supplemented with 2% FCS (Hyclone). T cells from spleens were enriched using T cell enrichment columns and then along with LN cells, applied to CD4 cell–enrichment columns (R&D Systems). The recovered CD4-enriched cells were labeled with PE–anti-CD25 antibody and incubated with anti–PE beads (Miltenyi Biotec), and then passed through a magnetic column according to the manufacturer's instructions. The recovered cells were then labeled with anti-CD4 antibody and sorted for either $CD4^+$ $CD25^-$ or $CD4^+$ $CD25^+$ T cells using a high speed cell sorter (MoFlo; DakoCytomation). $CD4^+$ $CD25^+$ T cell

contamination of sorted $CD4^+$ $CD25^-$ T cells was consistently <0.3% (Fig. 1 A). $10 \times 10^6$ sorted $CD4^+$ $CD25^-$ T cells from B.6SJL-$Ptprc^aPep3^b$/BoyJ mice ($CD45.1^+$) were then injected i.v. into either nonirradiated or sublethally irradiated (550 rads) C57BL/6 ($CD45.2^+$) mice. For some experiments, $CD45.1^+$ $CD4^+$ $CD25^-$ cells were labeled in complete media with 20 μM CFSE for 30 min at 37°C and then washed three times. And for some experiments, adult mice (6–8 wk of age) were thymectomized before receiving the i.v. injection of cells. Blood was collected weekly, and LN, spleen, and thymus (when available) were collected either 1 or 6 wk after injection. Cells were then either analyzed by FACS or sorted for use in in vitro assays or for analysis of Foxp3 expression. Absolute cell numbers were determined by direct cell counts and flow cytometric analysis.

**In vitro proliferation assay.** T cells from spleens were enriched using T cell–enrichment columns and then along with LN cells, applied to CD4 cell–enrichment columns (R&D Systems). CD4-enriched cells were labeled with anti-CD45.1, anti-CD4, and anti-CD25 and sorted by a high speed cell sorter. $CD4^+$ $CD25^+$ T cells were evaluated for their ability to suppress proliferation by coculture with $CD4^+$ $CD25^-$ responder T cells (either 25,000 or 12,000), irradiated spleen cells ($10^5$ cells) as APCs, and 10 μg/ml anti-CD3 antibody. (The regulatory/responder ratios that were tested in these assays were dependent on the number of converted $CD4^+$ $CD25^+$ cells recovered, i.e., under some experimental conditions, very few transferred cells were recovered.) For some experiments, anti-TGFβ, anti–IL-10, or 10–50 μg/ml of isotype antibody was added to the cultures. For the anergy experiments, 25,000 cells to be tested were cultured with irradiated spleen cells and anti-CD3 antibody as described above. Cells were cultured in complete media (RPMI 1640, 10% heat-inactivated FCS, 2 mM glutamine, 10 mM Hepes, 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and $10^{-5}$ M 2-mercaptoethanol) at 37°C and 5% $CO_2$ for 3 d. 0.5 μCi [$H^3$]thymidine was added for the last 18 h. Cells were harvested and [$H^3$]thymidine incorporation was measured by scintillation counter.

**Real-time PCR.** $CD4^+$ $CD25^+$ T cells were sorted to >98% purity. Total RNA was extracted using the Picopure RNA isolation kit (Arcturus) and reverse transcribed using the TaqMan reverse transcriptase kit (Applied Biosystems). The cDNA was amplified in duplicate by real-time PCR using the SYBR Green PCR kit (Applied Biosystems) with primers for GAPDH and Foxp3. Foxp3 mRNA levels were normalized relative to GAPDH mRNA expression. Data are presented as the fold-change relative to $CD4^+$ $CD25^-$ T cells. Primer pairs were designed using software provided by Applied Biosystems and synthesized and purified by HPLC by Integrated DNA Technologies. Primer pairs were as follows: Foxp3, 5′-CCCACCTACAGGCCCTTCTC-3′ and 5′-GGCATGGGCATCCACAGT-3′; GAPDH, 5′-GGAGCGAGACCCCACTAACA-3′ and 5′-ACATACTCAGCACCGGCCTC-3′.

**Statistical analyses.** Data were subjected to analyses by either the student's *t* test or ANOVA and the Tukey-Kramer multiple comparisons test. All experiments were performed at least twice, and the majority were performed three or more times with similar results.

**Online Supplemental Material.** Spleen cells from mice that have received $CD4^+$ $CD25^-$ cells were labeled with anti-CD45.1, CD4, and CD25 antibodies and analyzed for the presence of converted $CD4^+$ $CD25^+$ cells by FACS (Fig. S1). 25,000 converted $CD4^+$ $CD25^+$ cells were tested for their ability to proliferate in response to stimulation in vitro (Fig. S2). Converted $CD4^+$ $CD25^+$ cells harvested from $B7.1/B7.2^{-/-}$ mice 1 wk after transfer were labeled with anti-CD45.1, CD4, CD25, and either anti-GITR, CTLA-4, CD103, CD62L, or CD45RB antibodies and analyzed by FACS (Fig. S3). Total numbers of cells were calculated by using cell counts of LN and spleen and the results of FACS analysis (Table S1). Figs. S1–S3 and Table S1 are available at http://www.jem.org/cgi/content/full/jem.20041201/DC1.

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