Specific contribution of p19ARF to nitric oxide-dependent apoptosis

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CORRECTIONS


In Fig. 3B, the IAP-1 blot for wild-type (WT) cells is duplicated in error in the column for p53−/− macrophages. The corrected Fig. 3 is shown below.


The sixth institution in the author affiliations is incorrect. The corrected list is shown below.


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The third author’s last name is incorrect. The correct name is Suren R. Sooranna.


The second author’s name is incorrect. The correct name is Gabriela Pereira-da-Silva.


Changes that the authors did not request were made in production after the authors returned their page proofs resulting in publication of the article with multiple errors. The editors and staff of *The Journal of Immunology* apologize to the authors and readers for this error.

The entire article is reproduced correctly on the following pages in print only. The errors have been corrected in the online version, which now differs from the print version as originally published.
A Kinetic and Dynamic Analysis of Foxp3 Induced in T Cells by TGF-β1

Ramesh K. Selvaraj and Terrence L. Geiger

TGF-β induces Foxp3 expression in stimulated T cells. These Foxp3+ cells (induced regulatory T cells (iTreg)) share functional and therapeutic properties with thymic-derived Foxp3+ regulatory T cells (natural regulatory T cells (nTreg)). We performed a single-cell analysis to better characterize the regulation of Foxp3 in iTreg in vitro and assess their dynamics after transfer in vivo. TGF-β up-regulated Foxp3 in CD4+Foxp3+ T cells only when added within a 2- to 3-day window of CD3/CD28 stimulation. Up to 90% conversion occurred, beginning after 1–2 days of treatment. Foxp3 expression strictly required TCR stimulation but not costimulation and was independent of cell cycling. Removal of TGF-β led to a loss of Foxp3 expression after an ~4-day lag. Most iTreg transferred into wild-type mice down-regulated Foxp3 within 2 days, and these Foxp3+ cells were concentrated in the blood, spleen, lung, and liver. Few of the Foxp3+ cells were detected by 28 days after transfer. However, some Foxp3+ cells persisted even to this late time point, and these preferentially localized to the lymph nodes and bone marrow. CXCR4 was preferentially expressed on Foxp3+ iTreg within the bone marrow, and CD62L was preferentially expressed on those in the lymph nodes. Like transferred nTreg and in contrast with revertant Foxp3+ cells, Foxp3+ iTreg retained CD25 and glucocorticoid-induced TNFR family-related gene. Thus, Foxp3 expression in naïve-stimulated T cells is transient in vitro, dependent on TGF-β activity within a highly restricted window after activation and continuous TGF-β presence. In vivo, a subset of transferred iTreg persist long term, potentially providing a lasting source for regulatory activity after therapeutic administration. The Journal of Immunology, 2007, 178: 7667–7677.

Immunological tolerance is achieved developmentally in the thymus as well as through peripheral mechanisms. CD4+ regulatory T cells (Treg) that express the forkhead transcription factor Foxp3 are critical for maintaining peripheral tolerance; their deficiency leading to early-onset, fatal autoimmune inflammation (1). Foxp3 expression is not only a marker for Treg, but demonstrates that iTreg development requires TGF-β, which plays a critical role in their homeostasis (12, 13). Although deficiency in TGF-β dominantly leads to an autoimmune phenotype, the immunomodulatory role of TGF-β is likely complex. TGF-β also plays a crucial role in promoting the development of inflammatory Th17 cells and plays a supportive role in Th1 T cell development (14, 15).

Several reports have demonstrated utility in manipulating disease states by altering Treg numbers or activity in animal models (16–20). Increasing the number of Treg by adoptive transfer can diminish pathologic inflammation. Acquiring adequate numbers of Treg for treatment, however, represents a significant challenge due to the small number of nTreg present in vivo and their anergic state (21). An alternative and potentially simpler approach is to produce Foxp3+ iTreg from CD4+ Foxp3+ T cells by stimulation with TGF-β, which may then be used as an immunotherapeutic surrogate for nTreg. Indeed, iTreg are able to suppress T cell responses in vitro (22), educate allogeneic CD4+CD25+ cells to be suppressive (5), and have shown significant potential in the treatment or prevention of graft rejection, colitis, and diabetes in animal models (23–25).

The therapeutic applicability of iTreg will not only depend on their expression of Foxp3, but on other cellular characteristics. For instance, one leading hypothesis is that Treg development is guided by a high avidity for self-Ag (26, 27). iTreg, being derived from Foxp3+ T cells, lack this high avidity, which may influence their homeostatic or other properties. Differences between thymically derived nTreg and ex vivo-generated iTreg are not well studied. To better characterize iTreg, we have used single-cell analysis to assess the kinetics and sustainability of Foxp3 after induction with TGF-β in vitro and the cellular dynamics of iTreg in vivo. We demonstrate that iTreg development requires TGF-β exposure.
within a narrow window after stimulation and that Foxp3 persistence requires continued exposure to TGF-β. After adoptive transfer, iTreg predominantly and rapidly revert to Foxp3+ T cells. A subset of cells, however, retain Foxp3 for a longer time. These cells primarily localize to and possibly expand within the bone marrow and lymph nodes. We conclude that for the majority of iTreg, Foxp3 expression is transient and in vitro is highly dependent on exogenous TGF-β exposure. Some cells, however, develop stable expression of Foxp3 in vivo and phenotypically resemble nTreg.

Materials and Methods

Animals

Mice in which a GFP-Foxp3 fusion has been homologously inserted at the Foxp3 locus have been described (1) and were backcrossed more than five generations onto the C57BL/6J (Thy.1.2+, CD45.2+) background before analysis. Male mice screened for GFP-Foxp3 were used for experimentation. Some GFP-Foxp3 mice were subsequently bred with CD45.1-congenic mice to obtain CD45.1+ GFP-Foxp3 mice. C57BL/6J mice and congenic CD45.1 (B6.SL-Ptgsc Foxp3/YBoy) and Thy.1.1 (B6.PL-Thy1.2/CyJ) mice were purchased from The Jackson Laboratory. Experimentation was performed in accordance with institutional animal care and use procedures.

Media, reagents, and Abs

Media for T cell cultures was prepared as described earlier (28). Unconjugated anti-CD3, anti-CD28, and anti-CD16/CD3D Fc block (2.4G2) and fluorochrome-conjugated anti-CD4 (1L3T4), anti-CTLA-4, and anti-CXCR4 were purchased from BD Pharmingen. All other Abs used and recombinant human TGF-β1 were purchased from eBioscience. Sulfate latex beads (Molecular Probes and Invitrogen Life Technologies) were coated with anti-CD3 (40 μg/ml) or anti-CD28 (40 μg/ml) or anti-CD3/CD28 (13.3:26.6 μg/ml) as per the manufacturers’ instruction.

Cell purification and cell culture

Lymph nodes (LN) and spleen cells were collected as described previously (28). CD4+Foxp3+ (nTreg) and CD4+Foxp3− (non-Treg) cells were isolated by flow cytometric sorting on a MoFlo high-speed sorter (DakoCytomation) gating on CD4 and GFP (Foxp3) expression (28). Sorted cell purity ranged from 97 to 99%. nTreg were grown in medium supplemented with 1 ng/ml PMA, 200 ng/ml ionomycin, and 100 U/ml recombinant human IL-2 (rhIL-2; National Cancer Institute Biological Resources Branch Repository), which we found to optimally preserve Foxp3 expression. CD4+Foxp3− cells were stimulated with anti-CD3/CD28 beads at a cell:bead ratio of 1:1 and supplemented with 100 U/ml rhIL-2. At 48 h, murine IL-2, IL-4, IL-5, IL-10, IL-17, IFN-γ, and TNF-α were measured in the cell culture supernatant by Bio-Plex according to the manufacturer’s instructions (Bio-Rad).

Foxp3 regulation in CD4+Foxp3− cells

Sorted CD4+Foxp3− cells (2.5 × 10^6 per well) were stimulated in 96-well plates in medium supplemented with anti-CD3/CD28-coated beads (cell:bead ratio of 1:1) and 100 U/ml rhIL-2, or as indicated. TGF-β (10 ng/ml) was added at day 0 or the indicated time after TCR stimulation. For Foxp3 reversion kinetics, cells were stimulated as described above and TGF-β was removed at the indicated time by removing the supernatant and washing with PBS three times before adding back IL-2-containing medium. To study Foxp3 up-regulation in memory T cells, CD4+CD45RBlowFoxp3− and control CD4+Foxp3− cells were isolated by flow cytometric sorting, and 2 × 10^6 cells/well cultured in 96-well plates in medium supplemented with anti-CD3/CD28-coated beads (cell:bead ratio of 1:1) and 100 U/ml rhIL-2, with or without TGF-β (10 ng/ml). To prevent overcrowding in longer-term cultures, wells were examined and split every 3–4 days into medium supplemented with the same cytokines (IL-2 with or without TGF-β) as initially present. Quadruplicate samples for each condition were analyzed. Foxp3 analyses were performed every 24 h or as indicated by quantitative flow cytometry by measuring the GFP fluorescence.

PKH26 proliferation analysis

Naïve flow cytometrically purified CD4+Foxp3− cells were dye labeled using the PKH26 red fluorescent cell linker kit (Sigma-Aldrich) as per the manufacturer’s instructions, except that 1 × 10^6 M PKH26 dye was used for 1 × 10^6 cells. Cells were cultured in medium supplemented with or without TGF-β (10 ng/ml) and analyzed at the indicated times by flow cytometry.

T cell proliferation suppression assay

Flow cytometrically purified CD4+CD25− T cells from Thy.1.1-congenic mice (responder cells) were mixed with either nTreg or iTreg at the indicated ratios and stimulated with anti-CD3/CD28 beads at a bead:cell ratio of 1:1. Cocultures established in quadruplicate were analyzed by flow cytometry at the indicated times. For analysis of revertant Foxp3− iTreg populations, CD4+Foxp3− cells were converted to iTreg and sorted to >99% purity as described above. These iTreg were rewarmed with or without TGF-β for 5 days, at which time the cells were again flow-sorted for Foxp3+ or Foxp3− cells, respectively. The iTreg or revertant Foxp3− cells were then evaluated for their ability to suppress proliferation of CFSE-labeled naive T cell responders as previously described (29). Because the iTreg express GFP, the fluorescence emission spectrum of which cannot readily be distinguished from CFSE, Thy.1.1-disparate responder cells were used and analysis of CFSE levels in Thy.1.1-gated cells was performed.

Cytokine production profile of stimulated nTreg, iTreg, and non-Treg

CD4+Foxp3+ and CD4+Foxp3− cells were purified from GFP-Foxp3 knock-in mice and cultured as described above to obtain nTreg, iTreg, and non-Treg populations. Five × 10^6 cells were added to 96-well plates in 250 μl of medium in quadruplicate and restimulated with anti-CD3/CD28-coated beads at a 1:1 cell:bead ratio in the presence of 100 IU/ml rhIL-2. At 48 h, murine IL-2, IL-4, IL-5, IL-10, IL-17, IFN-γ, and TNF-α were measured in the cell culture supernatant by Bio-Plex according to the manufacturer’s instructions (Bio-Rad).

Migration and survival of iTreg after adoptive transfer

CD4+Foxp3+ and CD4+Foxp3− cells were purified from CD45.1+ GFP-Foxp3 knock-in mice and cultured as described above to obtain CD45.1+ GFP-Foxp3− iTreg and CD45.1+ Foxp3+ cells. At days 7–9, the expanded nTreg, iTreg, and non-Treg were flow-cytometrically sorted to obtain >99% pure Foxp3+ or Foxp3− populations. Five × 10^6 cells were added to 96-well plates in 250 μl of medium in quadruplicate and restimulated with anti-CD3/CD28 Ab-coated beads at a 1:1 cell:bead ratio in the presence of 100 IU/ml rhIL-2. At 48 h, murine IL-2, IL-4, IL-5, IL-10, IL-17, IFN-γ, and TNF-α were measured in the cell culture supernatant by Bio-Plex according to the manufacturer’s instructions (Bio-Rad).

Flow cytometry

Cells were stained and analyzed on a FACSCalibur (BD Biosciences) using CellQuest software (BD Biosciences). Quantitative flow cytometry to determine total cell numbers was performed by enumerating all cells in a culture well. The presence of Foxp3 in cells from mice expressing the GFP-Foxp3 fusion was determined by measuring GFP fluorescence.

Statistics

Data were analyzed by ANOVA using JMP (SAS).

Results

TGF-β collaborates with TCR stimulation to up-regulate Foxp3 in CD4+ cells

Previous studies (6–8, 30) have shown that TGF-β up-regulates Foxp3 in activated CD4+CD25− T cells. To directly visualize this event, we analyzed the induction of Foxp3 in T cells from mice engineered to express a GFP-Foxp3 fusion protein. CD4+Foxp3− T cells were purified, stimulated, and analyzed using quantitative flow cytometry. Similar to the results of others, TGF-β up-regulated Foxp3 in CD4+Foxp3− T cells after CD3/CD28 stimulation.
with conversion efficiencies of 50–90% routinely observed (Fig. 1, A and B). Costimulation influenced the conversion. Total numbers of Foxp3+ cells were increased in the presence of anti-CD28. However, the percentage of Foxp3+ cells was equivalent in cultures stimulated with anti-CD3 in the presence or absence of anti-CD28, suggesting that costimulation acted by promoting the expansion of the cells rather than increasing the conversion efficiency (Fig. 1B).

Foxp3 up-regulation was dependent on the presence of both TGF-β and TCR-specific signaling. As has been reported, in the absence of TGF-β, little or no up-regulation of Foxp3 was apparent (Fig. 1, A and B, and not plotted; after anti-CD3 and anti-CD3/CD28 stimulation, day 5 Foxp3+/Foxp3- cell counts were 212/95,567 and 128/141,065, respectively). In the absence of TCR stimulation, little conversion was also observed. This effect has been previously noted using RT-PCR analysis for Foxp3 (8). However, cell viability is extremely poor in the absence of TCR stimulation and an alternative explanation for this finding is that the Foxp3+ cells have impaired survival without TCR stimulation. Indeed, the total number of surviving T cells cultured with IL-2/TGF-β was only 1.1% of that observed in cultures also stimulated with anti-CD3/CD28. We found, however, that culture of CD4+ Foxp3- cells in the presence of anti-CD28 but not anti-CD3 greatly improved cell viability, with similar numbers of live cells present at day 5 as at the start of culture. Here too though, few of the cells (mean = 1.0%) up-regulated Foxp3. Therefore TCR but not CD28 signaling synergizes with TGF-β to drive Foxp3 expression. Foxp3 up-regulation was further restricted to the naïve T cell population and was not increased in isolated CD4+ CD44highCD45RblowFoxp3- memory cells stimulated in the presence of TGF-β (Fig. 1C).

**TGF-β-induced Treg suppress the proliferation of CD4+ target cells**

Earlier reports (7, 31) have shown iTreg, like nTreg, possess regulatory function and suppress CD4+ T cell proliferation in coculture experiments. We verified that our Foxp3+ iTreg were similarly capable of suppressing T cell expansion using quantitative flow cytometry (Fig. 2) as well as proliferation analysis of CFSE-labeled responder cells (data not shown). In both studies, iTreg showed an efficiency similar to that of nTreg in suppressing T cell proliferation and expansion.

**Similar cytokine profile of iTreg and nTreg**

Because iTreg were as efficient as nTreg in suppressing T cell proliferation, we wanted to examine whether their cytokine production profiles were likewise comparable (Table I). Naive CD4+ T cells were stimulated and expanded for 7 days without TGF-β or converted into iTreg with TGF-β. nTreg were likewise expanded. Foxp3+ (iTreg, nTreg) or Foxp3- (non-Treg) cells were then flow-cytometrically sorted and stimulated. Both iTreg and nTreg demonstrated decreased production of most cytokines when compared with non-Treg, including IL-2, IL-4, IL-5, IFN-γ, and

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**FIGURE 1.** TGF-β collaborates with TCR stimulation but not costimulation to up-regulate Foxp3. CD4+Foxp3- T cells were flow-cytometrically purified and 3 × 10^4 distributed per well of a 96-well plate. The cells were stimulated with anti-CD3, CD28, or CD3/CD28 Ab-coated beads at a 1:1 cell:bead ratio. rhIL-2 and TGF-β were added at 100 U/ml and 10 ng/ml, respectively. On pretreatment day 0 or posttreatment day 5, wells were harvested and analyzed for expression of Foxp3 by quantitative flow cytometry. Mean pretreatment values for Foxp3+ and Foxp3- T cells was 29,534 and 19, respectively, per well. A. Representative Foxp3 histogram plots are shown. B. Percent Foxp3+ cells is plotted. Mean absolute cell numbers are shown. C. Foxp3 is not up-regulated in memory cells. Cells were stimulated with anti-CD3/CD28-coated beads and IL-2 with or without TGF-β. Total cell counts on day 7 for the different populations are listed within parentheses. B and C. Mean ± SEM of quadruplicate samples are plotted.
TNF-α. Although more IL-2 and IFN-γ was produced by the iTreg than nTreg, this was significantly diminished when compared with non-Treg. In contrast, IL-10 was strongly produced by both the iTreg and nTreg. Therefore, TGF-β-induced iTreg have a cytokine profile similar to that of nTreg, with strong expression of IL-10 and diminished expression of other effector cytokines.

**Kinetics of Foxp3 expression after anti-CD3/CD28 and TGF-β treatment**

Considering that memory T cells did not up-regulate Foxp3 in response to TGF-β (Fig. 1C), we were interested in defining the window period after activation during which T cells were susceptible to TGF-β. Indeed, a time dependence for the generation of regulatory cells using TGF-β has been previously reported (32, 33). To test for Foxp3 induction, we cultured CD4⁺Foxp3⁻ cells with anti-CD3/CD28 and IL-2, supplementing with TGF-β at different time points after stimulation (Fig. 3A). Two effects were notable. First, when TGF-β was provided at the time of TCR stimulation, up-regulation of Foxp3 protein only began after an ∼2-day delay. Interestingly, if TGF-β supplementation was provided at later time points after TCR stimulation, Foxp3 up-regulation was delayed by a similar ∼2-day period from the time TGF-β was administered. Second, treatment with TGF-β beginning up to 2 days after TCR stimulation had little impact on the ultimate percentage or number of Foxp3⁺ T cells in the culture. In contrast, cells treated with TGF-β on or after day 3 showed significantly (p < 0.01) diminished conversion into Foxp3⁺ cells. When treatment began on day 3, a peak conversion of only ∼20% of cells was observed compared with ∼80% with day 0 treatment. This difference did not result from an outgrowth of Foxp3⁻ T cells because total cell numbers were similar in the different treatment groups (Fig. 3B). Indeed, quantitative analysis demonstrated that absolute numbers of Foxp3⁺ cells were significantly (p < 0.01) higher in the cells treated with TGF-β starting days 0–2 compared with the cells treated after day 2. Therefore, TGF-β/TCR stimulation has a limited window during which it can up-regulate Foxp3, and TGF-β supplementation leads to up-regulation of Foxp3 protein only after a significant (∼2-day) lag period.

**TGF-β- and CD3-induced Foxp3 up-regulation is independent of cell cycling**

Differentiation of naive T cells into Th1 and Th2 cell types occurs only after multiple rounds of cell cycling, an event believed to be required to relieve epigenetic repression of lineage-specific genes (34). After a T cell is stimulated through the TCR, it begins to cycle ∼2 days after stimulation, consistent with the time frame for TGF-β-mediated up-regulation of Foxp3 (Fig. 3A). We therefore interested whether Foxp3 up-regulation only occurred in T cells that had divided. To test this, we labeled CD4⁺Foxp3⁻ cells with the cell membrane-associated red fluorescent dye PKH26 and stimulated them with anti-CD3/CD28 with or without TGF-β (Fig. 4). The fluorescence of PKH-26 is diminished with each cell division. In the absence of TGF-β, Foxp3 expression was not observed in divided or undivided cells. In contrast, Foxp3 was up-regulated in all cell populations treated with TGF-β, including

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**Table 1. Cytokine production pattern of nTreg, iTreg, and non-Treg cells**

<table>
<thead>
<tr>
<th>Cytokine (pg/ml)</th>
<th>nTreg</th>
<th>iTreg</th>
<th>non-Treg</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>3.9</td>
<td>249.9</td>
<td>11,542.4</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.8</td>
<td>7.1</td>
<td>3,614.6</td>
</tr>
<tr>
<td>IL-5</td>
<td>0.5</td>
<td>3.1</td>
<td>46.8</td>
</tr>
<tr>
<td>IL-10</td>
<td>578.5</td>
<td>2,135.0</td>
<td>501.4</td>
</tr>
<tr>
<td>IL-17</td>
<td>2.3</td>
<td>19.2</td>
<td>27.6</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>3.6</td>
<td>487.9</td>
<td>2,544.7</td>
</tr>
<tr>
<td>TNF-α</td>
<td>2.7</td>
<td>4.8</td>
<td>36.2</td>
</tr>
</tbody>
</table>

*Five × 10⁶ flow-cytometrically sorted cells of the indicated type from 7-day cultures were added to 96-well plates in 250 μl of medium in triplicate and stimulated with anti-CD3/CD28-coated beads at a 1:1 cell/bead ratio. Mean cytokine production measured 48 h after stimulation is shown.*
To determine whether iTreg require continuous exposure to TGF-β, we were interested in whether loss of Foxp3 also leads to a loss of suppressive activity. To test this, TGF-β was either added to or excluded from cultures of flow-cytometrically purified Foxp3+ iTreg. Five days later, Foxp3+ or Foxp3- cells from the respective cultures were flow-cytometrically isolated. The iTreg or revertant Foxp3- cells were then added to naive, Thy1.1-disparate, CFSE-labeled T cells, and the proliferation of the naive population to anti-CD3/CD28 was measured by loss of CFSE (Fig. 6). As in Fig. 2, iTreg that retained Foxp3 strongly suppressed naive cell proliferation. In contrast, iTreg that lost Foxp3 showed a substantially reduced ability to suppress T cell proliferation. Thus, loss of Foxp3 expression is accompanied by a loss of suppressive activity.

Migration and survival of iTreg after adoptive transfer

Our in vitro studies suggested that the persistence of iTreg is dependent upon exogenous TGF-β and that Foxp3 is lost within a several-day period after TGF-β removal. The transient nature of Foxp3 expression in iTreg implies that iTreg would not be suitable for immunotherapeutic application. Yet, studies have now documented that iTreg are effective in treating model autoimmune diseases (23–25). To determine whether iTreg persist in vivo, we adaptively transferred flow-cytometrically purified GFP-Foxp3+ iTreg derived from CD45.1+ mice into CD45.1- congenic recipients and followed their migration and survival (Fig. 7). As controls, equivalent numbers of either Foxp3+ cells from those that had not divided. This demonstrates that Foxp3 up-regulation and iTreg development does not require cell cycling.

Limited persistence of Foxp3 in iTreg after TGF-β withdrawal

To determine whether iTreg require continuous exposure to TGF-β to preserve Foxp3 expression, we stimulated CD4+ Foxp3- cells with anti-CD3/CD28 and TGF-β, and withdrew TGF-β at various time points after stimulation (Fig. 5A). Foxp3 expression persisted at high levels in cells that received continuous TGF-β treatment for the 13 days of culture. In contrast, Foxp3 expression returned to baseline by day 13 in cells that had TGF-β removed after initial treatment. After TGF-β was withdrawn, Foxp3 persisted for another 3–4 days, at which point expression began to decrease (p = 0.013). Expression was virtually undetectable in the T cells by 9 days after TGF-β withdrawal. Cells that were exposed to <3 days of TGF-β lost Foxp3 at a much higher rate than those exposed for at least 3 days. Because total cell numbers in the wells were similar with or without withdrawal of TGF-β and total numbers of cells steadily increased with culture time (Fig. 5B), the loss of Foxp3 seemed to be primarily due to the conversion of the Foxp3+ cells to Foxp3- cells rather than cell death. This conversion was confirmed in separate experiments in which iTreg purified by flow cytometric sorting for GFP-Foxp3 and then cultured without exogenous TGF-β displayed a similar loss of Foxp3 (data not shown). When sorted Foxp3- iTreg were allowed to lose Foxp3 expression, Foxp3 could not be reinduced by restimulation, even in the presence of TGF-β (data not shown). Therefore, Foxp3 can only be induced in a brief window as naive T cells differentiate into effector/memory cells. Foxp3 persistence is dependent on sustained TGF-β signaling.

Loss of suppressive potency in iTreg that have down-regulated Foxp3

Considering that removal of TGF-β resulted in loss of Foxp3 in iTreg (Fig. 5A), we were interested in whether loss of Foxp3 also
cultures stimulated in the absence of TGF-β or Foxp3+ nTreg from similarly expanded cultures were also transferred into naive mice. The CD45.1-congenic background permitted identification of the transferred cells regardless of their expression of GFP-Foxp3. To control for adoptive transfer efficiency among mice, control freshly purified Thy1.1+/GFP−Foxp3− cells were CFSE labeled, mixed with the Foxp3+ or Foxp3− populations at the indicated ratios, and stimulated with anti-CD3/CD28-coated beads. At 72 h, cultures were stained for Thy1.1, and CFSE expression on the Thy1.1+ population was analyzed by flow cytometry.

At selected time points mice were sacrificed, cell suspensions were prepared from different organs, and the cells were analyzed by flow cytometry for CD4, Thy1.1, CD45.1, and Foxp3 expression. Similar numbers of Thy1.1+ cells were routinely observed in the recipient mice, indicating equivalent adoptive transfer efficiencies (data not shown). CD4+ cells were gated to distinguish the CD45.1+ transferred population and CD45.1− host cells, and the transferred cells were then further segregated into Foxp3+ and Foxp3− populations (Fig. 8, A and B). Numbers of CD45.1+ Foxp3+ or Foxp3− cells were normalized to endogenous CD4+ CD45.1−Thy1.1− cell numbers and plotted (Fig. 8C).

Transferred flow-cytometrically purified Foxp3+ iTreg largely disappeared within the first 2 days after adoptive transfer (Fig. 8C). This seemed to result from down-modulation of Foxp3 since large numbers of CD4−CD45.1+Foxp3− cells were simultaneously observed in several organs, including the spleen, liver, blood, and lung. Indeed, in support of this interpretation, numbers of Foxp3+ cells detected after transfer of purified Foxp3+ iTreg were similar to those observed after adoptive transfer of equal numbers of purified CD4+CD45.1+Foxp3− non-Treg T cells. Transferred CD4+Foxp3− cells were also found in similar locations as Foxp3+ former iTreg, specifically the spleen, liver, blood, and lung, within the first week after adoptive transfer. By 4 wk after transfer, virtually all of the Foxp3− cells from either the iTreg or control non-Treg transfer had disappeared. One organ where Foxp3 was retained on the transferred iTreg was the LN. Indeed, of the organs analyzed at day 2 after iTreg transfer, greater numbers of transferred Foxp3+ cells were only detected within the LN. This seemed to result from down-modulation of Foxp3 since large numbers of CD4−CD45.1+Foxp3− cells were observed, particularly in the bone marrow and LN. Interestingly, only small numbers of Foxp3+ cells remained in the spleen, suggesting differential localization or inadequate support of Foxp3 expression at this site. Because the transferred...
CD45.1+ cells were ~99% Foxp3+ (Fig. 7C), this indicates that a subset of iTreg persisted and possibly expanded, surviving for at least 4 wk and primarily localizing within the bone marrow and LN.

The survival and localization of iTreg showed significant differences though also similarities with that of adoptively transferred preactivated nTreg. nTreg retained their Foxp3 to a much greater extent than iTreg. All organs analyzed showed a predominance of Foxp3+ cells after nTreg transfer, yet this was only observed for the LN after iTreg transfer. Similar to the Foxp3+ cohort after iTreg transfer, the nTreg persisted 4 wk after transfer and at late time points also resided predominantly in the LN and bone marrow. One additional feature of nTreg transfers was that despite the transfer of equivalent numbers of nTreg as non-Treg, fewer nTreg were detected in the different organs than non-Treg. For example ~3,000 and 4,000 non-Treg were identified in the LN and spleen per 25,000 endogenous CD4+ T cells on day 2 after transfer, whereas ~400 and 130 nTreg were detected, respectively. This may reflect an increased rate of death or decreased proliferation of the preactivated nTreg vs non-Treg after transfer, or alternatively differential migration to other organs not analyzed. In summary, transferred Foxp3+ iTreg yielded large numbers of Foxp3+ cells that entered the blood, lungs, and liver and then disappeared by 2–4 wk after transfer. A portion of iTreg, however, retained Foxp3. These persevered initially in the LN and then, as for nTreg, increasingly in the bone marrow. Thus, although many iTreg act in vivo as in vitro, losing Foxp3 after removal from TGF-β, a subset of these cells persist longer as Foxp3+ iTreg.

**Transferred iTreg phenotypically resemble nTreg**

nTreg constitutively express several T cell activation markers, including CD25 and glucocorticoid-induced TNFR family-related gene (GITR) (35). These markers were also nearly uniformly up-regulated on both iTreg and activated non-Treg before adoptive transfer (Table II).

### Table II. Surface marker expression on iTreg, nTreg, and non-Treg cells

<table>
<thead>
<tr>
<th>Surface Marker</th>
<th>nTreg</th>
<th>Foxp3+</th>
<th>nTreg</th>
<th>iTreg</th>
<th>Non-Treg</th>
</tr>
</thead>
<tbody>
<tr>
<td>GITR</td>
<td>89.9</td>
<td>2.2</td>
<td>100</td>
<td>98.3</td>
<td>99.9</td>
</tr>
<tr>
<td>CD25</td>
<td>88.6</td>
<td>5.1</td>
<td>99.3</td>
<td>96.6</td>
<td>98.8</td>
</tr>
<tr>
<td>CD62L</td>
<td>38.2</td>
<td>55.2</td>
<td>83.2</td>
<td>26.0</td>
<td>29.8</td>
</tr>
<tr>
<td>CXCR4</td>
<td>2.2</td>
<td>1.3</td>
<td>2.9</td>
<td>2.8</td>
<td>1.9</td>
</tr>
</tbody>
</table>

*Freshly isolated CD4+ Foxp3+ or Foxp3+ cells were stimulated as described for 7–8 days to obtain Foxp3+ iTreg, Foxp3+ non-Treg, and Foxp3+ nTreg. Expression of the indicated markers before and after culture is shown.
To determine whether expression of CD25 and GITR persisted after adoptive transfer, cells from mice receiving activated and expanded iTreg, nTreg, and non-Treg were identified within the LN of recipient mice and analyzed for Foxp3 expression as in Figs. 7 and 8. These were additionally analyzed for surface expression of CD25 (A) or GITR (B). Percentage of CD4^+ CD45.1^+ cells of the indicated cell type positive for CD25 or GITR is plotted. Data points are missing for some cell types/conditions at some time points due to an inadequate number of cells in the organs at those times for definitive expression analysis. Sample histogram plots from the day 2 time point of transferred iTreg populations in LN that lost Foxp3 (left) or retained Foxp3 (right) are also shown. Mean ± SEM of two mice per condition are plotted. Data are representative of two independent experiments.

FIGURE 9. Transferred iTreg phenotypically resemble nTreg. CD4^+ CD45.1^+ adoptively transferred iTreg, nTreg, and non-Treg were identified within the LN of recipient mice and analyzed for Foxp3 expression as in Figs. 7 and 8. These were additionally analyzed for surface expression of CD25 (A) or GITR (B). Percentage of CD4^+ CD45.1^+ cells of the indicated cell type positive for CD25 or GITR is plotted. Data points are missing for some cell types/conditions at some time points due to an inadequate number of cells in the organs at those times for definitive expression analysis. Sample histogram plots from the day 2 time point of transferred iTreg populations in LN that lost Foxp3 (left) or retained Foxp3 (right) are also shown. Mean ± SEM of two mice per condition are plotted. Data are representative of two independent experiments.

FIGURE 10. CXCR4 is preferentially expressed on adoptively transferred cells in the bone marrow and CD62L on cells in the LN. Data were acquired and analyzed as in Figs. 7 and 8. A, Percentage of indicated adoptively transferred populations positive for CXCR4 on day 5 after transfer is plotted. Sample histogram plots demonstrating CXCR4 expression on Foxp3^- iTreg in the LN or bone marrow (BM) are also shown. B, Percentage of the indicated adoptively transferred population positive for CD62L on day 5 or day 12 after transfer is plotted. Sample histogram plots demonstrating CD62L expression on Foxp3^- iTreg in the LN or bone marrow are also shown. Mean ± SEM of two mice per condition are plotted. Data are representative of two independent experiments. ND, No data.
Foxp3 (Fig. 8) also lost surface expression of CD25 and GITR (data not shown). This indicates that Foxp3− iTreg phenotypically resemble nTreg in vivo, while the revertant Foxp3− iTreg resemble Foxp3+ cells. Foxp3 protein expression is associated with the sustained expression of CD25 and GITR in vivo.

It was interesting that the greatest concentration of transferred activated Foxp3+ iTreg or nTreg were found in the bone marrow and LN. Bone marrow localization of transferred nTreg has been previously shown to be associated with expression of the CXCR4 chemokine receptor, which is also expressed on other cell types localizing within the marrow (36). Indeed, blockade of CXCR4 can mobilize cells from the marrow into the blood (37). Analogously, nTreg expression of CD62L, a receptor for glycan ligands expressed on high endothelial venules, is associated with LN homing of nTreg as well as other T cells (38). To determine whether receptor expression correlates with tissue localization of iTreg, as it does for nTreg, we analyzed CXCR4 and CD62L expression on the transferred cells. At the time of transfer, <3% of iTreg expressed CXCR4 and only 26% expressed CD62L (Table II). By 5 days after transfer, ~90% of Foxp3+ iTreg or nTreg in bone marrow expressed CXCR4, while the same population in other organs showed very little CXCR4 expression (Fig. 10A). Similar results were seen at later time points (data not shown). This is consistent with CXCR4 being the bone marrow homing receptor for iTreg, as for nTreg.

At day 5, CD62L was discriminately expressed on cells within the LN; however, the differential expression was not as dramatic as for CXCR4 (Fig. 10B). By day 12, segregation of cells based on CD62L expression was more prominent. Approximately 80% of LN Foxp3+ cells (both nTreg and iTreg) expressed CD62L at day 12 (Fig. 10C), whereas only ~10% of Foxp3− cells in the bone marrow expressed CD62L. This indicates that the CD62L+ Foxp3+ iTreg population preferentially localizes to LN. Thus, adoptively transferred iTreg segregated into populations with distinct localization patterns that correlate with the expression of specific chemokine receptors or adhesion molecules.

**Discussion**

It is now well established that TGF-β can up-regulate Foxp3 in CD4+ Foxp3+ T cells. Using mice in which a GFP-Foxp3 fusion protein has been homologously inserted into the Foxp3 locus, we have been able to assess on a single-cell basis the kinetics of up-regulation of Foxp3 in vitro, as well as the maintenance of Foxp3 expression in vitro and in vivo. Our in vitro data are consistent with and adds to previous results. Foxp3 protein was up-regulated only after ~48 h of TGF-β treatment. This lag period in single-cell protein expression corresponds to a similar ~48-h lag in Foxp3 mRNA expression observed in human T cells (7). TGF-β-induced Smad activation and nuclear translocation occurs within minutes, and SMAD-DNA complexes can be observed as early as 10 min after TGF-β addition (39). This ~2-day delay suggests that Foxp3 up-regulation involves a complex developmental program rather than an immediate effect of SMAD-DNA interactions. Indeed, recent identification of the involvement of CTLA-4 and cbl-b in TGF-β-induced Foxp3 up-regulation (40, 41) supports the idea that complex combinatorial or sequential signals are involved in Foxp3 induction.

We further find that the lag in Foxp3 expression is independent of the time from initial TCR stimulation as when TGF-β is added 24 or 48 h after stimulation, Foxp3 up-regulation shows an identical ~2-day delay. This implies that a TCR-dependent factor is not rate-limiting in inducing Foxp3 protein expression, but rather TGF-β induces a sequence of events requiring this time frame to up-regulate Foxp3. Up-regulation does not require T cell cycling and is therefore unlikely to involve cell cycle-dependent modifications.

We find that TCR stimulation is needed for Foxp3 expression. Viability of naïve T cells cultured in the absence of TCR stimulation is poor and this viability loss may prevent examination of Foxp3 up-regulation in unstimulated populations. By stimulating cells with anti-CD28 in the absence of anti-TCR Ab, we were able to preserve viability and thereby clearly demonstrate this requirement. At 48–72 h after TCR stimulation, however, T cells become refractory to the effects of TGF-β. Therefore, a TCR-induced program is initially required for, although later restricts the ability of TGF-β to up-regulate Foxp3.

Previous studies have led to different conclusions on the role of CD28 costimulation in Foxp3 up-regulation. One study showed a marked diminishment in Foxp3 mRNA up-regulation after TGF-β induction of peripheral T cells stimulated in the presence of anti-CD28 Abs (7). In contrast, a second study found that CD28 costimulation was important for Foxp3 expression in TGF-β-treated CD4+ CD25− thymocytes, primarily by enhancing cell survival (42). In multiple experiments using highly purified Ab-stimulated T lymphocytes, we failed to observe either a beneficial or deleterious role for CD28 costimulation in Foxp3 up-regulation. The percentage conversion of Foxp3− T cells to Foxp3+ cells was similar regardless of the presence of costimulation. However, costimulation increased total cell numbers of both Foxp3− and Foxp3+ cells, suggesting that in our system it primarily promotes cell survival or proliferation rather than conversion per se. One important role of CD28 costimulation is the induction of IL-2 production in activated T cells (43). Since our cultures contained exogenous IL-2, which is an important facilitator of Foxp3 induction, this role of costimulation on Foxp3 up-regulation may have been masked in our system. Indeed, recent studies have emphasized the requisite role for IL-2 in Foxp3 up-regulation and in one case supported a role for CD28-induced IL-2 in this regard (44, 45).

We further analyzed the sustainability of Foxp3 after removal of TGF-β. Interestingly, continued Foxp3 expression in vitro requires continued exposure of iTreg to exogenous TGF-β. Foxp3 is lost from cells beginning several days after TGF-β removal and is eventually fully lost. Whether endogenous TGF-β expression can replace this exogenous TGF-β in sustaining Foxp3 is unclear. In our system, this was not the case. Data showing that TGF-β-induced T cells can educate naïve T cells to develop suppressive properties in a TGF-β- and IL-10-dependent manner, however, suggest that under some conditions a self-sustaining process promoting continued Treg development may take place (5). In those experiments, persistent Ag stimulation was required to sustain regulatory function and Foxp3 expression, and it may be hypothesized that this difference in stimulation conditions results in the induction of TGF-β.

The down-regulation of Foxp3 in iTreg was accelerated when the iTreg were adoptively transferred in vivo. By day 2 after transfer, few of the cells retained Foxp3. Interestingly, the tissue localization of Foxp3− former-iTreg was similar to that of transferred control Foxp3− T cells and different from that of transferred Foxp3− nTreg (Fig. 8). The reversion of iTreg into Foxp3+ T cells has potentially significant implications for their therapeutic utility because it may limit the intrinsic regulatory activity of the transferred cells. Indeed, we observed that iTreg that lost Foxp3 had diminished regulatory activity in a T cell proliferation suppression assay. In contrast to transferred iTreg, transferred activated nTreg showed limited loss of Foxp3 expression. Therefore iTreg and nTreg, although similar in suppressive function in vitro, differentially preserve Foxp3 after adoptive transfer.
Maintenance of nTreg requires several factors, including TGF-β and IL-2, in vivo (13, 46, 47). nTreg that lack IL-2R or that are from TGF-β−/− mice have a diminished peripheral life span. The differential maintenance of Foxp3 in nTreg and iTreg may suggest that transferred nTreg and iTreg have different access to sites where these cytokines are produced. An alternative or complementary possibility may lie in the distinct TCR repertoire of iTreg and nTreg. nTreg contain a skewed representation of TCR that is biased toward high affinity for self when compared with Foxp3+ T cells (27, 48). This self-specificity may alter the interactions of nTreg with resident APC compared with Foxp3− T cells, leading to the distinct homeostatic properties of the different cell types. Because iTreg are derived from Foxp3− T cells, we conjecture that they will lack the inherent self-specificity present in nTreg. Further studies analyzing the repertoire of stable Foxp3+ iTreg vs revertant Foxp3− cells are ongoing to explore this possibility.

Despite the loss of Foxp3 in the majority of transferred iTreg, a proportion of cells maintain Foxp3. Interestingly these cells concentrated primarily in two sites, the bone marrow and the LN. The number of transferred Foxp3+ cells increases in these locations over a 4-wk time period, suggesting that they expand there. Indeed, by day 28 after transfer, the numbers of Foxp3+ iTreg in the bone marrow and LN are equivalent to those of similarly transferred nTreg. Cellular localization of Foxp3+ T cells follows adhesion molecule expression. CXCR4, which is associated with bone marrow localization in a variety of cell types, is prominently expressed in the bone marrow resident population. Indeed, recent data have demonstrated that purified and transferred CXCR4+ nTreg primarily localize to the bone marrow (49). Likewise, we find that the majority of Foxp3+ iTreg inhabiting the LN are CD62Lhigh, a marker with a well-established role in LN homing (38).

It would be anticipated that the distribution of adoptively transferred iTreg should mimic that of endogenous Treg. Endogenous Treg freely distribute, although modestly different proportions may be detected in different lymphoid compartments. However, even 1 mo after transfer, we observed a skewed distribution of transferred cells to the LN and bone marrow. Our data on CXCR4 and CD62L expression suggest that this is due to preferential homing and localization to these organs; however, preferential survival or expansion of Foxp3+ cells in these sites cannot be excluded. Interestingly, few nTreg or iTreg expressed CXCR4 before transfer (Table II). Whether de novo up-regulation of this receptor occurred in vivo or the localization of the transferred cells was predetermined by their receptor expression after in vitro stimulation will be important to examine. The homing and localization of adoptively transferred therapeutic populations may impact their therapeutic qualities and understanding these processes will be important for any future application of in vitro-expanded regulatory cells.

Like nTreg, iTreg that retained Foxp3 after adoptive transfer also maintained CD25 and GITR surface expression. Our results suggest that these Foxp3+ cells, which were primarily found in the LN and bone marrow, play an important role in the amelioration of immunopathologic diseases after iTreg transfer. However, defining the role of the different cell populations in regulating disease will be important and it remains to be determined whether revertant Foxp3− cells have regulatory properties as well. Analyzing this may be complex since the transferred cells may not uniquely possess regulatory function. In one study, transferred iTreg were able to induce regulatory activity in endogenous populations of T cells (23). We have observed a similar infectious tolerance with transferred nTreg (50).

In summary, we demonstrate that iTreg are dependent on TGF-β signaling for the preservation of Foxp3 expression. Loss of Foxp3 occurs rapidly after in vivo transfer, with a predominance of Foxp3+ cells appearing within 2 days. The brief life span of Foxp3 in most transferred iTreg contrasts with that of transferred nTreg, which largely preserve Foxp3 expression. Although a subset of Foxp3+ iTreg is maintained, the distinct cellular dynamic properties of the iTreg and nTreg populations may lead to differential effects on immune responses, and this, in addition to the impact of the large numbers of Foxp3+ cells forming after iTreg transfer, must be considered in potential applications of the different cell types. iTreg, although phenotypically homogeneous after expansion in vitro, further segregate into different populations based on their expression of different homing/adhesion molecules, including CXCR4 and CD62L. Deciphering the role of the multiple cell types generated after iTreg transfer in the induction and preservation of immune tolerance will be important for understanding their therapeutic potential.

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Disclosures

The authors have no financial conflict of interest.

References


