Induction of FOXP3 expression in naive human CD4⁺FOXP3⁻ T cells by T-cell receptor stimulation is transforming growth factor-β–dependent but does not confer a regulatory phenotype

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Thymic-derived natural T-regulatory cells (nTregs) are important for the induction of self-tolerance and the control of autoimmunity. Murine CD4⁺CD25⁺Foxp3⁻ cells can be induced to express Foxp3 after T-cell receptor (TCR) activation in the presence of transforming growth factor β (TGFβ) and are phenotypically similar to nTregs. Some studies have suggested that TCR stimulation of human CD4⁺CD25⁻ cells results in the induction of transient expression of FOXP3, but that the induced cells lack a regulatory phenotype. We demonstrate here that TCR stimulation alone was insufficient to induce FOXP3 expression in the absence of TGFβ, whereas high levels of FOXP3 expression could be induced in the presence of TGFβ. Although FOXP3 expression was stable, the TGFβ-induced FOXP3⁺ T cells were neither anergic nor suppressive and produced high levels of effector cytokines. These results suggest that even high levels of FOXP3 expression are insufficient to define a human CD4⁺ T cell as a T-regulatory cell. (Blood. 2007;110:2983-2990)

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Introduction

Thymic-derived natural T-regulatory cells (CD4⁺CD25⁺Foxp3⁺; nTregs) are important for the induction of self-tolerance and the control of autoimmune disease.1 Mutations in the FOXP3 transcription factor result in a rare human disorder called immune dysregulation/polyendocrinopathy/enteropathy/X-linked syndrome (IPEX), which is characterized by multiple autoimmune diseases.2,3 Patients with this condition typically die by early infancy or childhood. A similar condition consisting of a fatal lymphoproliferative disorder with multigorgan infiltration occurs in the scurfy mouse as a result of a deletion of the Foxp3 forkhead domain.4,5 Deficiencies in nTreg numbers or function have been postulated to play a role in susceptibility to several autoimmune diseases.6 It is essential to study and understand the functions of FOXP3⁺ nTregs, but human nTregs have proven difficult to isolate because CD25 cannot be readily used as a marker for FOXP3⁺ cells. The CD4⁺CD25⁺ population contains mostly cells that are FOXP3⁺ and express intermediate to low levels of CD25.7

Expression of Foxp3 is considered the definitive marker for mouse nTregs because Foxp3 is not induced during the activation of non–T-regulatory cells.5 Several studies have suggested that this is not the case in humans because T-cell receptor (TCR) stimulation alone with anti-CD3 and anti-CD28 has been reported to induce FOXP3 expression in human CD4⁺CD25⁻ cells.8-15 In one study, these cells expressed a T-regulatory phenotype,9 whereas several other studies demonstrated that the induction of FOXP3 did not correlate with anergy or suppressive function.10,14,15 There are several problems with these studies. In many, it was difficult to quantify the purity of the starting population, rendering it difficult to distinguish induction of FOXP3 from selection of a few FOXP3⁺ nTregs present in the starting population. Several studies determined the expression of FOXP3 after induction at the population level using quantitative PCR, whereas others measured FOXP3 at the single-cell level using an anti-FOXP3 monoclonal antibody (mAb), PCH101, that may give nonspecific staining.

A second potential difference between the regulation of FOXP3 expression in the mouse and human is that mouse CD4⁺CD25⁻Foxp3⁻ cells can be induced to express Foxp3 when activated in vitro by TCR stimulation in the presence of transforming growth factor β (TGFβ).16,17 TGFβ-induced mouse Tregs appear to resemble nTregs in all their phenotypic and functional properties. They exert potent suppressor function in vitro and can prevent or control disease in vivo.18,19 Only one study has suggested that TGFβ can induce FOXP3 in human CD4⁺CD25⁻ cells, but the analysis was at a population level and the function of the induced cells was not assessed.17

In this study, we re-examined the requirements for de novo induction of FOXP3 in nonregulatory human CD4⁺FOXP3⁻ T cells. Using single-cell analysis with multiple mAbs to FOXP3 as well as quantitative PCR, we demonstrate that human CD4⁺FOXP3⁻ cells resemble mouse CD4⁺Foxp3⁻ cells. TCR stimulation alone was insufficient to induce FOXP3 expression in the absence of TGFβ, although high levels of FOXP3 could be induced in the presence of TGFβ. FOXP3 expression in the TGFβ-induced cells was stable for several weeks in culture without TGFβ but did require the presence of interleukin-2 (IL-2). In contrast to TGFβ-induced mouse CD4⁺Foxp3⁺ cells that were both anergic and suppressive in vitro, human induced CD4⁺FOXP3⁺ cells were neither anergic nor suppressive and produced high levels of effector cytokines. The failure of human TGFβ-induced CD4⁺FOXP3⁺ cells to exhibit regulatory activity raises the
possibility that even high levels of FOXP3 expression are insufficient to define a cell as a Treg and that other factors, present in mouse CD4+ T cells, may be required to act in concert with FOXP3.

Materials and methods

Cell purification

Peripheral blood was obtained from healthy adult donors by the Department of Transfusion Medicine at the National Institutes of Health. Cord blood was collected from term placentas at Shady Grove Adventist Hospital (Gaithersburg, MD). The acquisition of blood products was approved according to the Institutional Review Boards (IRBs) of these 2 institutions and in accordance with the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were prepared over Ficoll-Paque Plus gradients (GE Healthcare, Piscataway, NJ). The CD4+ cells were enriched over the autoMACS separation column (Miltenyi Biotec, Auburn, CA). The cells were labeled with CD4 fluorescein isothiocyanate (FITC), CD45RA Tricolor, and CD25 APC purchased from Invitrogen (Carlsbad, CA) and CD127 phycoerythrin (PE) from BD Biosciences Pharmingen (San Diego, CA) and sorted with either the FACS Vantage DVer or FACS Aria flow cytometer (both from BD Biosciences, San Jose, CA). Likewise, the CD4+CD25hi cells were obtained by labeling the cells with CD4 FITC and CD25 PE and fluorescence-activated cell sorting on the top 2% of CD25hi. The postsort purity for CD4+CD25−CD127−CD45RA+ and CD4+CD25−CD127+CD45RA− cells was higher than 97% and the FOXP3 purity for CD4+CD25hi cells was higher than 90%.

In vitro cell activation

The cells sorted by FACS were activated in vitro with plate-bound anti-CD3 and 2 μg/mL soluble anti-CD28 (BD PharMingen) with or without 5 μg/mL transforming growth factor β1 (TGFβ1; Peprotech, Rocky Hill, NJ) in 24-well culture plates (Corning Life Sciences, Acton, MA) at 500,000 cells per well. The culture medium consisted of X-VIVO 20 (Lonza, Walkersville, MD) with or without 5% heat-inactivated autologous serum and 50 IU/mL IL-2 (Hoffman-LaRoche, Nutley, NJ). The culture plate was coated overnight at 4°C with 10 μg/mL OKT3 (Ortho Biotech Products, Bridgewater, NJ). In some experiments, 50 μg/mL neutralizing anti-TGFβ antibody was used (clone 1D11; R&D Systems, Minneapolis, MN). The cells were stimulated for 5 days then washed and transferred to new wells with fresh culture medium containing 50 IU/mL IL-2. The cells were split and fresh culture medium replaced every 2 days. Otherwise, the CD4+CD25−CD127−CD45RA+ cells were activated with either CD3-depleted PBMC, immature monocyte-derived dendritic cells, or mature monocyte-derived dendritic cells in the presence of 1 μg/mL OKT3 and 50 IU/mL IL-2 with or without 5 μg/mL TGFβ1.

Generation of antigen-presenting cells

The CD3-depleted PBMC was generated by incubating PBMC with human CD3 microbeads (Miltenyi Biotec) and running it over the autoMACS with the depletion-sensitive program. The dendritic cells (DCs) were generated from monocyte-derived dendritic cells in the presence of 1 μg/mL soluble anti-CD28 (BD PharMingen) with or without 50 IU/mL IL-2 (Hoffman-LaRoche, Nutley, NJ). The day 5 cultures were additionally supplemented with 5 ng/mL TGFβ1 and 50 IU/mL IL-2 (Hoffman-LaRoche, Nutley, NJ). The culture medium was higher than 97% and the FOXP3 purity for CD4+CD25hi cells was higher than 90%.

FACS analysis

FACSCalibur was used for data acquisition, and the data were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR). For staining of FOXP3, the cells were fixed and permeabilized using a Fixation/Permeabilization kit according to the manufacturer’s protocol (eBioscience, San Diego, CA). The FOXP3 was stained with either PCH101 PE (eBioscience) or 259D Alexa Fluor 647 (Biologend, San Diego, CA) anti-FOXP3 antibody and isotype control.

For analysis of intracellular cytokine production, CD45RA+ cells activated on day 5 were rested in 50 IU/mL IL-2 culture medium for 2 days and restimulated for 5 hours with 25 ng/mL phorbol 12-myristate 13-acetate (PMA) and 250 ng/mL ionomycin (Sigma-Aldrich), along with 3 μg/mL brefeldin A (eBioscience). Afterward, the cells were fixed and permeabilized with an eBioscience kit and stained for FOXP3 with 259D Alexa Fluor 647 and for cytokines with anti-interferon γ (IFNγ) FITC, IL-2 PE, and IL-10 PE purchased from Invitrogen.

Quantitative real-time polymerase chain reaction analysis

Total RNA was extracted from cells with an RNeasy Plus Kit (Qiagen, Valencia, CA). Reverse transcription-polymerase chain reaction (RT-PCR) was performed with approximately 1 μg of isolated RNA for cDNA synthesis using SuperScript II RNase H-reverse transcriptase (Invitrogen). Real-time PCR was performed in triplicate according to the TaqMan Universal 2X master mix and run on the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). The amount of FOXP3 mRNA expression was normalized to the 18S rRNA and calculated according to the comparative cycle threshold (Ct) method as described by Applied Biosystems. FOXP3 primers and probe (Integrated DNA Technologies, Coralville, IA) were as follows: forward primer, 5'-GCA CCT TCT CAA ATC CCA GT-3'; reverse primer, 5'-GGGC CAT TTG CAG ACA CCA T-3'; and fluorogenic probe (5-carboxyfluorescein/5-carboxytetramethylrhodamine), 5'-CAG GAA GGA CAG CAC CCT TTC GGC-3'.

FOX3 siRNA experiments

FOX3 siRNA was purchased from Invitrogen (Stealth Select RNAi). FOX3 oligonucleotide HSS121456 was used as nonsilencing siRNA and oligonucleotide HSS121458 was used as silencing siRNA. To transfect the CD45RA+ cells, 200 pmol of siRNA was mixed with 100 μL of the human T-cell Nucleofector solution and 5 × 105 cells were reseeded in this mixture. The cell suspension was immediately electroporated over the Nucleofector II instrument (Amaxa Biosystems, Gaithersburg, MD). The transected CD45RA+ cells were rested in 50 IU/mL IL-2 culture medium for 24 hours before being stimulated with anti-CD3/anti-CD28 and 50 IU/mL IL-2 in the presence of 50 μg/mL anti-TGFβ or 5 μg/mL TGFβ1 for 5 days and stained for FOX3 with PCH101.

FOX3 stability experiments

The CD45RA+ cells stimulated on day 5 were rested in 50 IU/mL IL-2 culture medium for 2 days then washed and replaced with fresh IL-2 culture medium with or without 50 μg/mL anti-TGFβ. Otherwise, the cells were placed in culture medium without IL-2 and 10 μg/mL anti-IL2 (clone 5334; R&D Systems) along with either 50 ng/mL IL-1β, TNFα, IFNγ, IL-4, IL-6, IL-7, IL-10, IL-15 (Peprotech) or without any cytokine. After 48 hours, the cells were stained for FOXP3 with 259D.

Proliferation assays

CD45RA+ cells were activated with or without TGFβ1 and CD4+CD25+ nTregs were activated without TGFβ1 for 5 days and rested for 2 days in IL-2 culture medium. The cells were washed 3 times in phosphate-buffered saline before using them for the suppression assay. Their suppressive functions were tested on day 7 with 5 × 104 fresh allogeneic CD4+CD25+ responder cells, sorted by FACS, stimulated with 5 × 104 irradiated (4000 rads) autologous CD3-depleted PBMC. and 0.5 μg/mL OKT3 alone or with non-TGFβ-treated CD45RA+, TGFβ-treated CD45RA+, activated CD4+CD25hi, or fresh CD4+CD25hi cells. The cells were cultured for...
Results

Induction of FOXP3 expression in naive human CD4+ T cells was TGFβ-dependent

To better define the roles of TCR stimulation and TGFβ in the induction of FOXP3, we purified naive human CD4+CD25−CD127+CD45RA+ T cells by FACS. These cells will be referred to as CD45RA+. We used the CD127 marker to minimize any contaminating CD4+CD25−FOXP3+nTregs that have been shown to express low to absent level of CD127.20 The resultant population contained less than 0.1% FOXP3+nTregs as assessed by staining with mAb 259D (Figure 1A). After in vitro stimulation for 5 days with anti-CD3/anti-CD28 and IL-2 in culture medium containing 5% autologous serum, approximately 30% of the cells expressed FOXP3, and this percentage could be increased to approximately 80% in the presence of exogenous TGFβ1. Five-day stimulation gave optimal induction of FOXP3 expression. When the cells were stimulated in the presence of a neutralizing anti-TGFβ mAb, less than 4% of the cells expressed FOXP3. TGFβ1 had no effect on the increase in the level of FOXP3 as defined by mean fluorescence intensity (MFI) in CD4+CD25+nTregs (Figure 1B). The level of FOXP3 induced in the CD45RA+ cells was never as high as that seen in the stimulated nTregs but was higher than that observed in unstimulated nTregs.

These results suggest that the induction of FOXP3 in the absence of exogenous TGFβ is mediated by TGFβ present in the serum or produced by the responding T cells. When CD45RA+ cells were stimulated in serum-free medium, approximately 5% of the cells expressed FOXP3 and the addition of anti-TGFβ had little effect (Figure 1C). In contrast, when exogenous TGFβ1 was added to the serum-free medium, more than 70% of the stimulated cells expressed FOXP3. These data are consistent with the results of studies with naive mouse CD4+ T cells that have shown that TCR activation alone does not induce Foxp3 and that the induction of Foxp3 requires TGFβ.

Memory T cells were resistant to TGFβ-induced FOXP3 expression

To determine whether all CD4+FOXP3− cells could be induced to express FOXP3, we compared the susceptibility of CD4+CD25−CD127+CD45RA+ and CD4+CD25−CD127+CD45RA− cells to the induction of FOXP3 with TGFβ. It was again important to analyze only CD127+ T cells, in that the majority of the nTregs are CD45RA−CD127− cells.21 The postsort level of FOXP3 was consistently less than 0.1% for the CD45RA+ and less than 1% for the CD45RA− population. Upon activation of these cells with anti-CD3/anti-CD28, IL-2, and TGFβ1 for 5 days, approximately 75% of the CD45RA+ population expressed FOXP3 (Figure 2A). In contrast, only approximately 15% of the CD45RA+ population could be induced to express FOXP3 with TGFβ. Similar results were observed from 30 different donors with an average of 22% (range, 6%-42%) of the CD45RA+ cells expressed FOXP3 without exogenous TGFβ and an average of 75% (range, 60%-91%) expressed FOXP3 in the presence of TGFβ1 (Figure 2B). In contrast, only an average of 16% (range, 3%-28%) of the CD45RA− cells expressed FOXP3 without exogenous TGFβ and an average of 28% (range, 14%-45%) expressed FOXP3 with TGFβ. Because naive CD4+ cells were more susceptible to the induction of FOXP3 by TGFβ1 and to minimize any potential contaminating FOXP3−CD25− nTregs, we used the CD4+CD25−CD127+CD45RA+ population for all of our experiments.
Anti-FOXP3 mAb PCH101 was nonspecific for human FOXP3

Our results (ie, that only approximately 22% [range, 6%-42%] of CD45RA+ cells could be induced to express FOXP3 without exogenous TGFβ) are contrary to those of several published studies, indicating that the majority expressed FOXP3 when stained with anti-FOXP3 mAb PCH101.13-15 To investigate this discrepancy, activated CD45RA+ cells were stained in parallel with PCH101 and 259D. Based on the isotype control, almost all of the cells activated without TGFβ expressed FOXP3 when stained with PCH101, and 2 peaks of different intensities were seen when the cells were cultured with exogenous TGFβ (Figure 3A). When analyzed with 259D and its isotype control, only a few cells expressed FOXP3 when stimulated without TGFβ, and more than 60% expressed FOXP3 with exogenous TGFβ. Two other anti–human FOXP3 mAbs, 206D and 236A/E7, gave results similar to those seen with 259D (data not shown).

To verify that activated cells stained nonspecifically with PCH101, we also measured FOXP3 expression by quantitative PCR. In the presence of anti-TGFβ, the level of FOXP3 mRNA and protein in CD45RA+ cells stimulated on day 5 was very low and was similar to that detected in the starting population of CD45RA+ cells (Figure 3B). When the CD45RA+ cells were stimulated with exogenous TGFβ1, there was a greater than 100-fold increase in FOXP3 mRNA. However, the level of FOXP3 mRNA and protein in the activated TGFβ-treated CD45RA+ cells was always less than activated nTregs but higher than unstimulated nTregs.

Similar conclusions could be drawn when CD45RA+ cells were transfected with nonsilencing or silencing FOXP3 siRNA and then stimulated in the presence or absence of TGFβ. The reactivity of PCH101 with the cells activated without TGFβ was not reduced by the FOXP3 siRNA (Figure 3C left panel). Furthermore, when the cells were stimulated with exogenous TGFβ, only the bright peak stained with PCH101 was reduced by siRNA treatment, whereas the less bright population was not susceptible to the siRNA and resembled the level of reactivity seen without TGFβ (Figure 3C center and right panels). Taken together, these studies indicate that PCH101 is an unreliable indicator of FOXP3 expression in human activated T cells and gives a false conclusion that FOXP3 represents an activation marker on all activated human CD4 T cells.13,15

TGFβ-induced FOXP3 expression was stable

When FOXP3 expression was induced by stimulation of CD45RA+ cells with TGFβ for 5 days and the cells were then washed and...
maintained in IL-2 culture medium, the percentage of cells staining for FOXP3 was remarkably stable over a 30-day period (Figure 4A). The continued presence of IL-2 was important, in that neutralization of IL-2 over a 48-hour period resulted in a decrease in the percentage and MFI of FOXP3+ cells (Figure 4B). No decrease in cell viability was seen during this period. In contrast, neutralization of TGFβ, in the presence of IL-2, had no effect on the percentage of FOXP3+ cells. It is noteworthy that other cytokines (IL-4, IL-7, IL-15) that use the common γ-chain as part of their receptor complex were also able to maintain FOXP3 expression in the absence of IL-2, whereas IL-1β, TNFα, IFNγ, IL-6, and IL-10 had no effect.

**TGFβ-induced FOXP3+ T cells were neither anergic nor suppressive**

The phenotypic characteristics of the TGFβ-induced FOXP3+ cells were very similar to thymic-derived nTregs. They stably expressed both isoforms of FOXP3, down-regulated expression of CD127, and up-regulated expression of CTLA-4 (data not shown). To evaluate their regulatory potential, CD45RA+ cells were activated in vitro with TGFβ for 5 days and rested in IL-2 culture medium for 2 days. FACS-purified CD4+CD25hi nTregs also were activated for 5 days and rested for 2 days in IL-2. On day 7, freshly isolated allogeneic CD4+CD25− cells were stimulated with autologous CD3-depleted PBMC and anti-CD3 in the presence of non-TGFβ-treated CD45RA+, TGFβ-treated CD45RA+, activated CD25hi nTregs, or freshly isolated CD25hi nTregs. The percentage and levels of expression of FOXP3 in these populations are shown in the top panel of Figure 5A. Both activated and fresh nTregs failed to proliferate when stimulated with anti-CD3 and were potent inhibitors of the proliferative responses of CD4+CD25− cells as assayed by [3H]thymidine incorporation and CFSE dilution (Figure 5). In marked contrast, the TGFβ-treated FOXP3+ cells were similar to the non–TGFβ-treated FOXP3− cells in that they proliferated when stimulated and failed to suppress responder CD4+CD25− cells. We have allowed these TGFβ-treated FOXP3+ cells to be rested in IL-2 culture medium until day 14, but they still lacked suppressive and anergic functions (data not shown).

**TGFβ-induced FOXP3+ T cells produced IL-2 and IFN-γ**

One of the problems in the interpretation of the above studies is that FOXP3 expression was only seen in approximately 75% to 80% of the TGFβ-treated CD45RA+ cells. It remains possible that cytokine production by the FOXP3+ cells was inducing proliferation of the FOXP3+ cells and masking their anergic state and their suppressive capacity. We were unable to increase the percentage of FOXP3+ cells by sorting on CD25 or CD127, because both FOXP3+ and FOXP3− CD45RA+ cells from the TGFβ-stimulated cultures expressed similar levels (data not shown). We therefore restimulated the TGFβ-treated CD45RA+ cells with PMA and ionomycin and examined cytokine production at the single cell level by intracellular staining. Although the activated CD4+CD25hi nTregs were anergic, both the FOXP3+ and FOXP3− CD45RA+ cells from the TGFβ-stimulated cultures produced significant amount of IFN-γ and IL-2 (Figure 6).

It is also possible that the CD45RA+ cells present in adult peripheral blood are not truly naïve and are in part resistant to some of the inductive effects of TGFβ or to FOXP3-mediated T-regulatory functions. We therefore isolated cord blood CD45RA+ T cells and induced FOXP3 expression using the same protocol described for adult CD45RA+ T cells. Although the majority of the cord blood in CD45RA+ cells could be converted to FOXP3+ cells, they were neither anergic nor suppressive (data not shown), and a significant percentage of the FOXP3+ population produced IFN-γ and IL-2 (Figure 6).

**Induction of FOXP3 in CD45RA+ cells activated with antigen-presenting cells required TGFβ but was insufficient to induce T regulatory functions**

To determine whether other methods of T cell activation could influence the induction of FOXP3 and T regulatory functions, the CD45RA+ cells were stimulated for 5 days with allogeneic immature dendritic cells (iDC), mature dendritic cells (mDC), or CD3-depleted PBMC (APC) in the presence of 1 μg/mL OKT3 and 50 IU/mL IL-2 with or without 5 ng/mL TGFβ1. Similar to stimulation with anti-CD3 and anti-CD28, TGFβ is required for induction of FOXP3 with iDC and APC (Figure 7A). It is noteworthy that mDC were not very efficient for inducing FOXP3 in CD45RA+ cells. One explanation is that the mDC could be secreting IL-6, which has been shown to inhibit the induction of FOXP3 by TGFβ.22 To test whether the TGFβ-induced FOXP3 expression in CD45RA+ cells stimulated with iDC and mDC was sufficient to suppress IL-2 production, these cells were restimulated with PMA and ionomycin. Similar to activation with anti-CD3 and
cells by TCR stimulation with anti-CD3 and anti-CD28 results in the induction of FOXP3 and Treg function. We have performed a careful single-cell analysis of the requirements for TCR stimulation and TGFβ costimulation in the induction of FOXP3 in human naive CD4+FOXP3-cells. Human CD4+FOXP3-cells can only be induced to express FOXP3 by TCR stimulation in the presence of TGFβ. FOXP3 expression remains stable in long-term cultures of the induced cells. IL-2, but not TGFβ, is required for maintenance of high levels of FOXP3 expression. One major difference between our studies and those of other groups is that we have used mAb 259D to analyze FOXP3. In our hands, mAb PCH101, the anti-FOXP3 reagent used in many human studies, results in false-positive staining, particularly of activated human CD4+ T cells. We have verified our results with other mAbs to FOXP3, by quantitative PCR analysis, and by siRNA knockdown studies.

After induction of Foxp3 in mouse T cells either by retroviral mediated transfection or TCR stimulation with TGFβ, expression of Foxp3 seems to be sufficient to induce most of the phenotypic and functional characteristics of thymic-derived nTregs. Previous studies have shown that transfection of human FOXP3 into naïve CD4+ T cells can induce Treg functions, although in one study,22 anergy but not suppression was observed. Our results indicate that TGFβ-mediated induction of FOXP3 was insufficient to confer anergy and suppressive function to human CD4+FOXP3-cells.

The TGFβ-induced FOXP3+ cells express both isoforms of FOXP3, up-regulate CTLA-4, and down-regulate CD127. However, the levels of both FOXP3 and CTLA-4 are considerably less than those seen on nTregs that have been stimulated under the same condition. Wan and Flavell have recently described a mutant mouse strain, the nTregs from which express attenuated levels of Foxp3, lack suppressive activities, become Th2 effector cells, but do remain anergic.23 Thus, the absolute levels of Foxp3 expressed by a cell may determine whether it exhibits a T regulatory phenotype. It thus remains possible that the levels of TGFβ-induced FOXP3 are simply too low to drive the T regulatory pathway. Transfection of FOXP3 may result in higher levels and perhaps more stable levels of expression. On the other hand, the FOXP3+ and FOXP3− CD45RA+ cells expressed a similar level of IL-2 (Figure 7B).

**Discussion**

Expression of Foxp3 has proven to be a reliable marker for mouse thymic-derived nTregs, for Tregs generated in peripheral lymphoid tissues by exposure to antigen under nonstimulatory conditions,23,24 and for those generated in vitro after TCR stimulation in the presence of TGFβ. In contrast, some studies have claimed that in vitro activation of human CD4+CD25− anti-CD28, both the FOXP3+ and FOXP3− CD45RA+ cells expressed a similar level of IL-2 (Figure 7B).
the other hand, the levels of FOXP3 on the induced cells are higher than those seen in freshly explanted CD4+CD25hi nTregs that fail to produce cytokines in short-term assays.

Multiple factors may control both the cellular susceptibility to induction of FOXP3 by TGFβ and the capacity of cells that express FOXP3 to manifest T regulatory cell activity. Transfection of FOXP3 was less efficient in reprograming human CD4+CD45RO+ memory T cell into regulatory cells,25 and Foxp3 could not be induced in recently activated mouse CD4+ T cells by restimulation in the presence of TGFβ.26 Most of our studies were performed with CD4+ T cells from normal adult donors, and it remains possible that the CD45RA+ cells in adult blood are not truly naive and in fact represent memory cells or experienced T cells that cannot respond to or lack components of the FOXP3 driven regulatory pathways. However, similar results were obtained with cord blood CD4+CD25−CD127+CD45RA+ cells. Human CD4+ T cells may also require costimulatory signals or cytokine signals in concert with TGFβ for induction of T regulatory cell function. We have used alternative methods of T cell activation with accessory cells, including CD3-depleted PBMC and monocyte-derived dendritic cells with results similar to those obtained only in the presence of plate-bound antibodies.

Human CD4+CD25− cells may lack critical downstream components of a FOXP3-driven suppressor pathway. It is now clear that interaction of Foxp3 with other transcription factors (NFAT or Runx-1)30,31 or modification of Foxp3 by differential histone acetylation was critical to its action.32 Other studies suggest that induction of some components of the T regulatory phenotype occurs before Foxp3 expression during T-cell development in the thymus and that the function of Foxp3 is to amplify and fix the molecular features of T-regulatory cells.33,34 CD4+Foxp3+ cells from the peripheral lymphoid tissues of young mice may retain certain components of the T regulatory pathway that can then be acted on and stabilized by induced Foxp3 to render them Tregs, whereas human CD4+FOXP3− cells may lack these molecules that are essential for the T regulatory phenotype.

One critical question that remains to be addressed is whether FOXP3 can still be considered a definitive marker for human Tregs. FOXP3 expression correlates well with T-regulatory function in CD4+CD25hi cells derived from normal donors. On the other hand, when the immune system has been perturbed, our studies suggest that considerable caution should be exercised before concluding that a FOXP3+ T cell is a bona fide Treg. It is still unclear whether TGFβ costimulation in vivo can lead to peripheral conversion of human CD4+CD25−FOXP3− to CD4+CD25hiFOXP3+ cells. However, TGFβ is found at high levels at inflammatory sites, and some of the CD4+CD25hiFOXP3+ cells isolated from such tissues may resemble the FOXP3+ non−T-regulatory cells we have generated in vitro. It has been claimed that Tregs from patients with several autoimmune diseases are deficient in their suppressive capacities in vitro and that this may be one of the fundamental factors that predispose to autoimmune disease.35–40 However, it is possible that some of the CD4+CD25hi cells isolated from patients may also represent TGFβ-induced FOXP3+ non−T-regulatory cells that contaminate the nTreg population and contribute to the defects in suppression that have been observed in these clinical studies. Based on our findings, another concern involves in vitro expansion of human nTregs, which is currently being considered for potential immunotherapy. It remains possible that some of the CD4+CD25hiFOXP3+ cells in the starting population are non−T-regulatory cells that had been induced in vivo in response to TGFβ, and these cells may preferentially expand in culture. Although the expanded cells might be all FOXP3+, their functions might be effector instead of regulatory. Likewise, the TGFβ in the serum can potentially induce FOXP3 expression in the few CD4+CD25hiFOXP3− cells found in the nTreg starting population and these cells would preferentially outgrow and contaminate the culture.

Finally, antigen-specific TGFβ-induced Foxp3+ murine T cells have proven to be highly effective in the prevention of autoimmune disease. Identification of other factors that might lead to the conversion of human FOXP3− T cells to FOXP3+ T regulatory cells remains an important area for future study. Autoantigen-specific patient-derived Tregs have been proposed for the cellular biotherapy of human autoimmune disease.41 The capacity to convert human autoantigen-specific effectors into Tregs in vitro may ultimately represent a more practical alternative.
Acknowledgments

We thank the NIAID Flow Cytometry Section, particularly Carol Henry, Tom Moyer, and Calvin Eigsti, for all their help in sorting our cells. We also thank Cynthia Matthews and Rosemary Werden in the Department of Transfusion Medicine for providing us with our cells. We also thank Cynthia Matthews and Rosemary Werden, Henry, Tom Moyer, and Calvin Eigsti, for all their help in sorting our cells. We thank the NIAID Flow Cytometry Section, particularly Carol Henry, Tom Moyer, and Calvin Eigsti, for all their help in sorting our cells.

References


Authorship

Contribution: D.Q.T. initiated and conducted all experiments, prepared the figures, and drafted the manuscript. H.R. helped with the FOXP3 real-time PCR experiments. E.M.S. (principal investigator) supervised the project and edited the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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