

# Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production

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**Keywords:** human, T cells, T cell activation, tolerance, transcription factors

## Abstract

Forkhead box P3 (FOXP3) is currently thought to be the most specific marker for naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> T regulatory cells (nTregs). In mice, expression of FoxP3 is strictly correlated with regulatory activity, whereas increasing evidence suggests that in humans, activated T effector cells (Teffs) may also express FOXP3. In order to better define the role of FOXP3 in human Teff cells, we investigated the intensity and kinetics of expression in *ex vivo* Teff cells, suppressed Teff cells and Teff cell lines. We found that all dividing Teff cells expressed FOXP3, but only transiently, and at levels that were significantly lower than those in suppressive nTregs. This temporary expression in Teff cells was insufficient to suppress expression of reported targets of FOXP3 repressor activity, including CD127, IL-2 and IFN- $\gamma$ , and was not correlated with induction of a nTreg phenotype. Thus expression of FOXP3 is a normal consequence of CD4<sup>+</sup> T cell activation and, in humans, it can no longer be used as an exclusive marker of nTregs. These data indicate that our current understanding of how FOXP3 contributes to immune tolerance in humans needs to be re-evaluated.

## Introduction

The forkhead box P3 (FoxP3) transcription factor has a critical role in the development of naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> T regulatory cells (nTregs), which are required for maintenance of immune tolerance. Mice lacking a functional *FoxP3* gene suffer from systemic autoimmunity, and evidence from adoptive transfer experiments suggests that this is the direct result of a deficit in nTregs. Moreover, ectopic expression of FoxP3 in mouse CD4<sup>+</sup> T cells is sufficient to generate nTregs *in vitro* (1–3), leading to the conclusion that expression of this single transcription factor causes a developmental switch to a suppressor cell phenotype. FoxP3 is thus considered a more definitive lineage marker of nTregs than CD25, and recent evidence that, in mice, cells that are CD4<sup>+</sup>FoxP3<sup>+</sup> but CD25<sup>−</sup> are suppressive, supports this concept (4).

Humans lacking a functional *FOXP3* gene also suffer from a systemic autoimmune disease known as immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome

(IPEX), and it was hypothesized that this disease was also due to the lack of nTregs (5). Surprisingly, however, we found that, depending on the type of mutation, IPEX patients can have normal numbers of nTregs that are suppressive under conditions of weak activation (6). In addition, retrovirus-mediated expression of full-length FOXP3, and/or a form of FOXP3-lacking exon 2 (FOXP3b), does not consistently convert human CD4<sup>+</sup> T cells into potent nTregs, suggesting that additional factors are required for FOXP3 to cause the full developmental switch to nTregs in humans (7–9).

The apparently different roles of FOXP3 in mice and humans may be related to the capacity of human non-regulatory CD4<sup>+</sup> T effector cells (Teffs) to express FOXP3 upon activation (7, 10–14). Early reports suggested that a proportion of human CD4<sup>+</sup> Teff cells always express FOXP3<sup>+</sup> when activated, and that these cells then become phenotypically and functionally indistinguishable from nTregs (10, 11). More recent studies suggest that expression of FOXP3 in human

Teff cells does not necessarily lead to a suppressive phenotype, since CD4<sup>+</sup>FOXP3<sup>+</sup> T cell clones that are not suppressive can be isolated (13, 14), and production of IL-2 and IFN- $\gamma$  is not suppressed in all FOXP3<sup>+</sup> Teff cells (12).

In order to further investigate the hypothesis that FOXP3 has a distinct role in Teff cells, we characterized the kinetics and levels of FOXP3 expression in activated *ex vivo* Teff cells, Teff cell lines and suppressed Teff cells. Here we report that FOXP3, like other conventional T cell activation markers, is transiently up-regulated in all dividing Teff cells, but at levels that are significantly lower than those in nTregs. This activation-induced expression in Teff cells is insufficient to uniformly suppress the expression of transcriptional targets of FOXP3, such as CD127, IL-2 and IFN- $\gamma$ . These data imply that FOXP3 has a role in human Teff cells that is independent of its capacity to promote nTreg development.

## Methods

### Cell purification

Peripheral blood was obtained from healthy volunteers following approval of the protocol by the University of British Columbia Clinical Research Ethics Board and after obtaining written informed consent from individual donors. CD4<sup>+</sup> T cells were purified from PBMCs by negative selection (StemCell, Vancouver, Canada). CD4<sup>+</sup>CD25<sup>+</sup> nTregs were purified by positive selection over two MS columns (purity  $\geq 90\%$ ) and CD4<sup>+</sup>CD25<sup>-</sup> Teff cells by depletion over LD columns (purity  $\geq 99\%$ ) (Miltenyi Biotec, Auburn, CA, USA). For generation of T cell lines, the brightest 1% of CD25<sup>+</sup> cells and the dimmest 20% of CD25<sup>-</sup> cells were FACS sorted from total CD4<sup>+</sup> T cells on a FACS Aria, and expanded *in vitro* as previously described (15). Antigen-presenting cells (APCs) were prepared by depletion of CD3<sup>+</sup> cells by positive selection (purity  $\geq 95\%$ ) (StemCell).

### Activation, proliferation and suppression experiments

Purified T cells were cultured in X-VIVO15 medium with 5% AB human serum (from Cambrex, Walkersville, Canada), 1 $\times$  penicillin and streptomycin and 1 $\times$  Glutamax (from Invitrogen, Burlington, Canada), in the absence of exogenous IL-2 unless indicated otherwise. For activation and proliferation experiments, purified T cells were plated in 48- or 24-well plates at a density of 1  $\times$  10<sup>6</sup> cells ml<sup>-1</sup> and stimulated with anti-CD3/CD28-coated beads (from Invitrogen, Burlington, Canada) in the absence of IL-2. Unless indicated otherwise, a bead to cell ratio of 1:16 was used as based on titration experiments required for each lot of beads. This ratio provided a moderate stimulation and typically resulted in 25–50% FOXP3<sup>+</sup> Teff cells at 72 h. Cells were split after 5 days to prevent overgrowth, and every 2 days thereafter. Alternatively, purified T cells were activated with plate-bound anti-CD3 (10  $\mu$ g ml<sup>-1</sup> OKT3, Ortho Biotech, Bridgewater, NJ, USA) in the presence or absence of different co-stimulatory molecules: IL-2 (100 U ml<sup>-1</sup>, Chiron, Ville Saint-Laurent, Canada), anti-CD28 (1  $\mu$ g ml<sup>-1</sup>, BD PharMingen, Mississauga, Canada), Pam-3-Cys (1  $\mu$ g ml<sup>-1</sup>, Axxora, San Diego, CA, USA) or *Escherichia coli* flagellin [100 ng ml<sup>-1</sup>, purified as described (16)]. In some experiments, freshly purified Teff

cells were first labeled with 2.5  $\mu$ M 5- (and 6-) carboxy-fluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR, USA) prior to setting up experiments. For suppression experiments, CFSE-labeled Teff cells were activated with irradiated (5000 RADS) autologous APCs at a 1:1 ratio in the presence of anti-CD3 (1  $\mu$ g ml<sup>-1</sup> OKT3, Ortho Biotech), in the presence or absence of nTregs for 96 h.

### Western blotting

A minimum of 3  $\times$  10<sup>6</sup> cells were used to make each T cell lysate. Nuclear and cytoplasmic extracts were enriched using an NE-PER nuclear and cytoplasmic extraction kit (Pierce Biotechnology Inc., Nepean, Canada). Protein concentrations were determined using a BCA assay and 7  $\mu$ g of protein was loaded per lane on 10% SDS-PAGE gels. Nitrocellulose membranes were probed with polyclonal rabbit anti-FOXP3 antiserum (17), followed by goat anti-rabbit-HRP (DakoCytomation, Mississauga, Canada). Membranes were stripped and reprobed with anti-TFIIIB (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) to ensure loading equivalency.

### Flow cytometric analysis

Staining for cell-surface markers CD69, CD25, CD127 (BD PharMingen) and glucocorticoid-induced tumor necrosis factor receptor (GITR) (R&D Systems, Minneapolis, MN, USA) was carried out prior to intracellular staining for cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (BD PharMingen) or FOXP3. Staining for FOXP3 was performed with PCH101 or 236A/E7 (eBiosciences, San Diego, CA, USA) according to the manufacturer's instructions. The results with 236A/E7 or PCH101 were equivalent. Based on recommendations from Herzenberg (18) and Roederer (19), gates for FOXP3-positive cells were set based on fluorescence minus one controls.

For analysis of intracellular cytokine production, purified T cells were first activated with anti-CD3/CD28-coated beads (from Invitrogen) or left unstimulated for 3 days. Cells were then washed, counted and left unstimulated or exposed to a secondary stimulation of phorbol myristate acetate (PMA) (10 ng ml<sup>-1</sup>, Sigma, Oakville, Canada) and Ca<sup>2+</sup> ionophore (500 ng ml<sup>-1</sup>, Sigma) for 4 h, with brefeldin A (10  $\mu$ g ml<sup>-1</sup>, Sigma) added for the final 2 h. Cells were fixed and stained for intracellular IL-2, IFN- $\gamma$  (BD PharMingen) and FOXP3 according to the manufacturer's protocols for FOXP3 staining. Samples were acquired immediately after staining on a BD FACSCanto and analyzed with FCS Express Pro Software Version 3 (De Novo Software).

### Statistics

All analyses for statistically significant differences were performed with 1-tailed paired Student's *t*-test. *P* values of <0.05 were considered significant.

## Results

### Expression of FOXP3 in resting versus activated CD4<sup>+</sup> T cell subsets

We and other groups have reported that in humans, expression of FOXP3 can be induced in Teff cells upon TCR-mediated

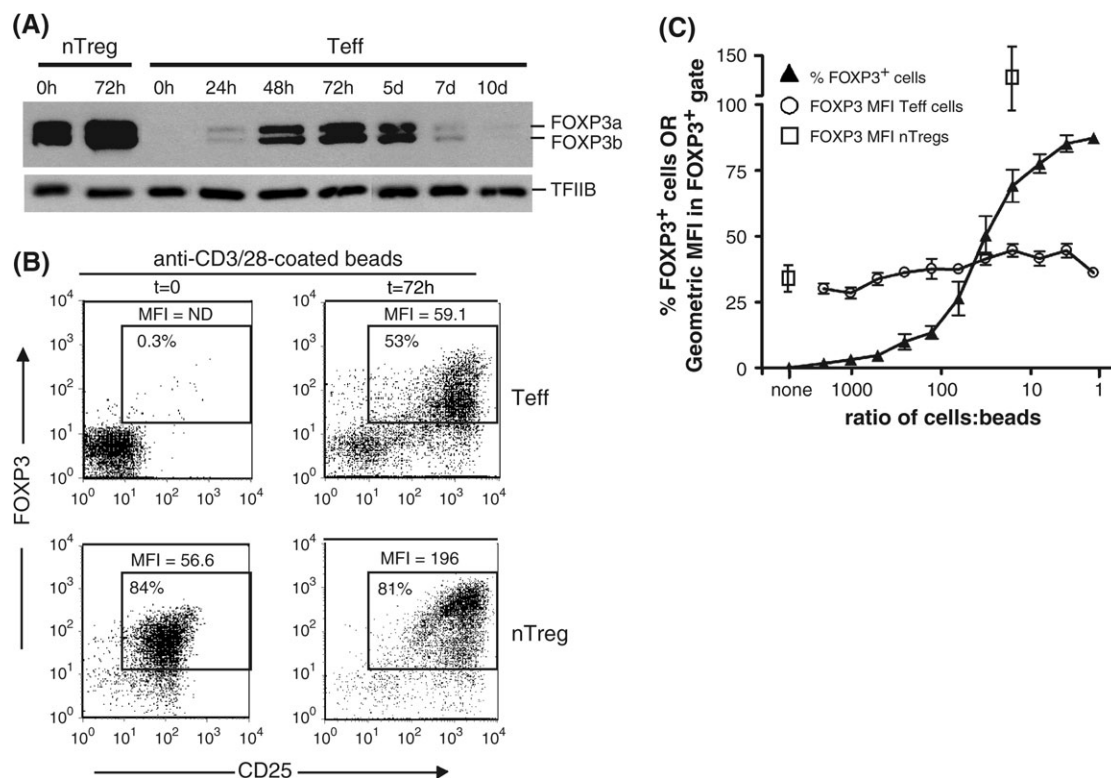
stimulation (7, 10–14, 20, 21). It is unclear, however, whether this activation-induced FOXP3 is associated with the *de novo* development of Tregs and suppressive activity (10–12) or simply a normal consequence of activation. To further investigate this question, we first performed a detailed comparison of the kinetics and intensity of FOXP3 expression in Tregs and Teff cells. Highly purified nTreg and Teff cells were activated with anti-CD3/CD28-coated beads and expression of FOXP3 was followed over time. The experiments were performed in the absence of exogenous IL-2 in order to determine the innate capacity of Teff cells to express FOXP3. Western blotting (Fig. 1A) demonstrated that both full-length FOXP3 (FOXP3a) and the smaller isoform which lacks exon 2 (FOXP3b) (7) were detectable in the nuclear fraction of activated Teff cells, with expression peaking at 72 h and then gradually declining. In nTregs, expression of FOXP3a/b also increased upon activation to levels greater than those observed in highly activated Teff cells. Flow cytometric analysis of resting and stimulated nTreg and Teff cells confirmed these results, with a significantly higher mean fluorescence intensity (MFI) of FOXP3 in nTregs than in Teff cells after 72 h of activation (Fig. 1B). The anti-FOXP3 mAbs used for flow cytometric analysis (clone PCH101 or 236A/E7) were found to recognize both isoforms of FOXP3 (data not

shown), thus detecting total FOXP3a/b (hereafter referred to simply as FOXP3).

We next investigated whether all CD4<sup>+</sup> Teff cells have the potential to express FOXP3, and compared the intensity of expression in Teff cells versus nTregs. Accordingly, Teff cells were activated with increasing numbers of anti-CD3/CD28-coated beads, and analyzed for expression of FOXP3. Surprisingly, under strong activation conditions, virtually 100% of Teff cells expressed FOXP3 (Fig. 1C). The total amount of FOXP3 protein induced per cell was similar to that in resting nTregs, but was always significantly lower than levels in nTregs exposed to equivalent activating conditions. These data suggest that in the absence of exogenous factors, Teff cells may have a molecular block that prevents them from expressing high levels of FOXP3, or that nTregs express a unique complement of proteins that drives elevated and sustained expression.

#### FOXP3 is an activation marker in Teff cells

In addition to FOXP3, nTregs constitutively express CD25, CTLA-4 and GITR, molecules that are also up-regulated on activated Teff cells. To investigate how the kinetics of activation-induced expression of FOXP3 in Teff cells compared with these conventional activation markers, we assessed their



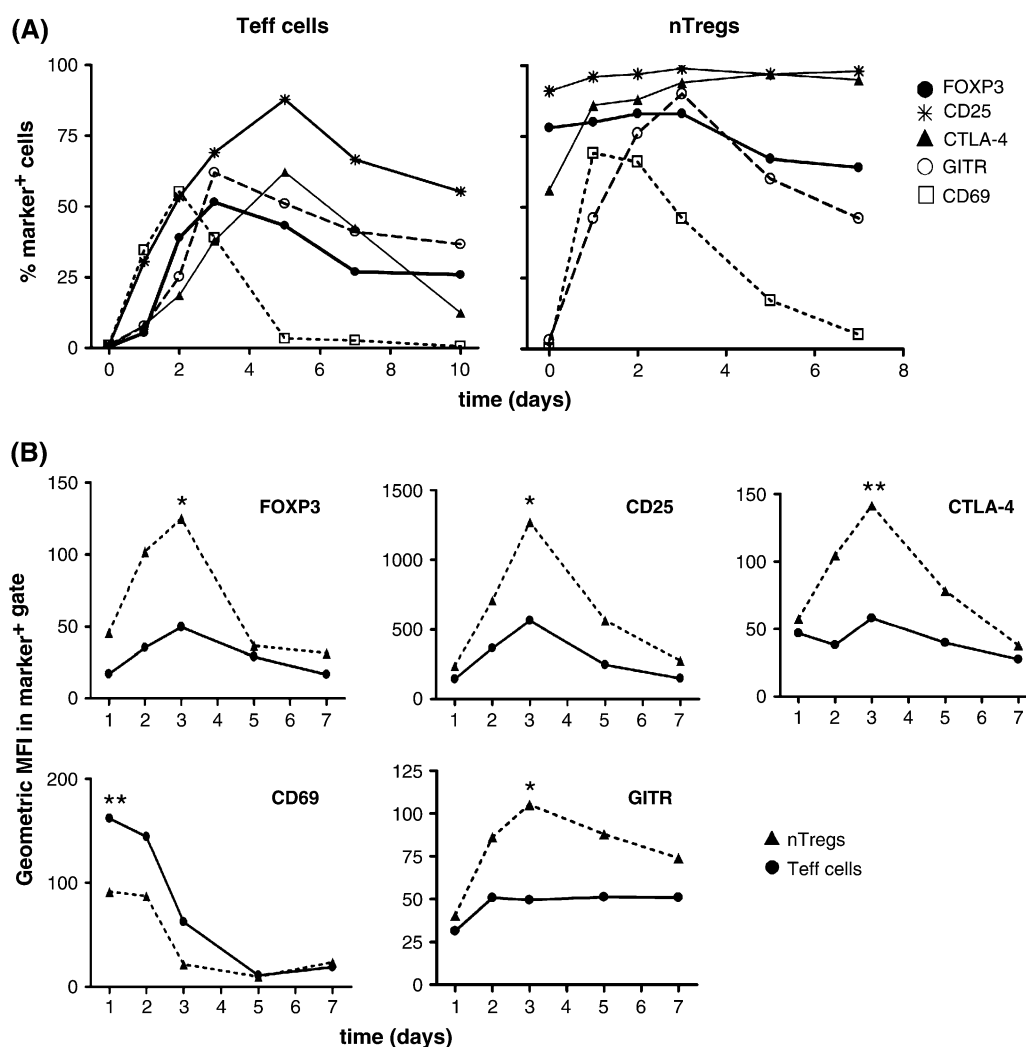
**Fig. 1.** Kinetics of activation-induced FOXP3 expression in Teff cells compared with nTregs. (A) *Ex vivo* nTreg and Teff cells were activated with anti-CD3/CD28-coated beads for the indicated times, and nuclear lysates were probed for expression of FOXP3 and TFIIB (as a loading control). (B) *Ex vivo* nTreg and Teff cells were activated as in (A) for 72 h and analyzed for CD25 and FOXP3 expression using anti-FOXP3-PE (clone PCH101). The MFI of FOXP3 was determined by gating on the FOXP3<sup>+</sup> cells as indicated. (C) *Ex vivo* Teff cells were activated with increasing numbers of anti-CD3/CD28-coated beads for 72 h and analyzed for FOXP3 expression using anti-FOXP3-PE (clone PCH101). The percent FOXP3<sup>+</sup> cells and MFI in the FOXP3<sup>+</sup> gate are shown. The MFI of FOXP3 expression of resting or activated nTregs is shown for comparison. (A) is representative of  $n = 2$ , (B)  $n = 8$  and (C)  $n = 5$  experiments.

expression in parallel over a period of 10 days. As expected, *ex vivo* Teff cells were FOXP3<sup>-</sup>, CD25<sup>-</sup>, CTLA-4<sup>-</sup>, GITR<sup>-</sup> and CD69<sup>-</sup>. Following activation, the peak of FOXP3, CTLA-4 and GITR expression occurred between 3 and 5 days (Fig. 2A), whereas CD69 and CD25 increased at earlier time points. After peaking at 72 h, FOXP3 expression gradually declined, with a parallel decrease in expression of CTLA-4 and GITR. It is unlikely that this decline in FOXP3<sup>+</sup> cells was simply due to cell death since even strongly activated cultures in which >90% of cells became FOXP3<sup>+</sup> (e.g. Fig. 1C) did not show significant cell death.

In parallel, the kinetics of expression of these markers was also determined in nTregs. *Ex vivo* cells purified on the basis of CD25 expression were typically 65–85% FOXP3<sup>+</sup>, 30–60% CTLA-4<sup>+</sup>, GITR<sup>low</sup> and CD69<sup>-</sup>. Upon activation, the percentage of nTregs expressing CTLA-4, GITR and CD69 increased. In contrast to Teff cells, the percentage of cells

expressing CD25, FOXP3 and CTLA-4 did not significantly decline over a period of 7 days.

To further characterize the pattern of activation-induced FOXP3 in Teff cells, we directly assessed the intensity of FOXP3 expression in purified populations of Teff cells and compared them with nTregs (Fig. 2B). In parallel, we analyzed expression of CD25, GITR and CTLA-4 as examples of other nTreg-associated markers, as well as CD69 as a control for T cell activation. Levels of expression of CD25, CTLA-4 and GITR increased in both cell types upon activation. The intensity of the activation-induced expression of FOXP3, CD25, CTLA-4 and GITR in nTregs was significantly higher than that in activated Teff cells. After 72 h, expression of FOXP3 in nTregs was 2.3-fold  $\pm$  0.77-fold higher than in Teff cells ( $P = 0.026$ ,  $n = 6$ ), that of CD25 1.83-fold  $\pm$  0.42-fold higher ( $P = 0.014$ ,  $n = 4$ ), that of CTLA-4 3.2-fold  $\pm$  1.1-fold higher ( $P = 0.001$ ,  $n = 5$ ) and that of GITR



**Fig. 2.** FOXP3 is an activation marker in Teff cells. *Ex vivo* Teff cells or nTregs were activated with anti-CD3/CD28-coated beads and analyzed for expression of (A) the percentage of cells positive for indicated molecules or (B) the MFI of marker<sup>+</sup> fractions over 7 days. Experiments with nTregs were limited to 7 days as in the absence of exogenous IL-2 viability was compromised at longer time points. Anti-FOXP3-PE staining was carried out with PCH101 and verified with 236A/E7. Statistical significance: \* $P < 0.03$  and \*\* $P < 0.001$ . Plots are representative of at least three independent experiments.

2.24-fold  $\pm$  0.46-fold higher ( $P = 0.032$ ,  $n = 3$ ). In contrast, CD69 was up-regulated to a greater extent in Teff cells than in nTregs (2.0-fold  $\pm$  0.35-fold higher,  $P = 0.0001$ ,  $n = 4$ ; at 24 h). In addition, while nTregs uniformly co-expressed these molecules (with the exception of CD69), not all FOXP3<sup>+</sup> Teff cells were CD25, CTLA-4 or GITR positive (data not shown).

#### Expression of FOXP3 in Teff cells is not dependent on signaling via CD28

It has been shown that co-stimulation via CD28 is necessary for the expression of Foxp3 in thymocytes and the development of nTregs (22). To investigate the co-stimulatory requirements for activation-induced FOXP3 expression, we stimulated highly purified Teff cells with immobilized anti-CD3 mAbs alone or in the presence of different co-stimulatory agents (Fig. 3). As a control, cells were stimulated with anti-CD3/CD28-coupled beads at a ratio expected to stimulate a moderate amount of FOXP3 expression. After 72 h, flow cytometric analysis demonstrated that stimulation via the TCR alone was sufficient to induce a small proportion of Teff cells to up-regulate FOXP3, while co-stimulation via CD28, TLR2 [with Pam-3-Cys (23)] or TLR5 [with flagellin (16)] led to an increase in the percentage of FOXP3<sup>+</sup> cells (Fig. 3A). A similar increase was observed in the presence of exogenous IL-2, suggesting co-stimulatory agents may enhance FOXP3 expression due to their capacity to promote endogenous IL-2 production. The proportion of FOXP3-expressing cells correlated with the number of cell divisions in each culture condition, as demonstrated by CFSE dilution after 5 days (Fig. 3B). Thus, weak stimulatory conditions that promoted minimal proliferation induced a low proportion of cells to express FOXP3, while strong activating conditions promoted significant cell division and FOXP3 up-regulation, irrespective of whether CD28 was activated. This observation is in agreement with a report from Gavin *et al.* (12), in which

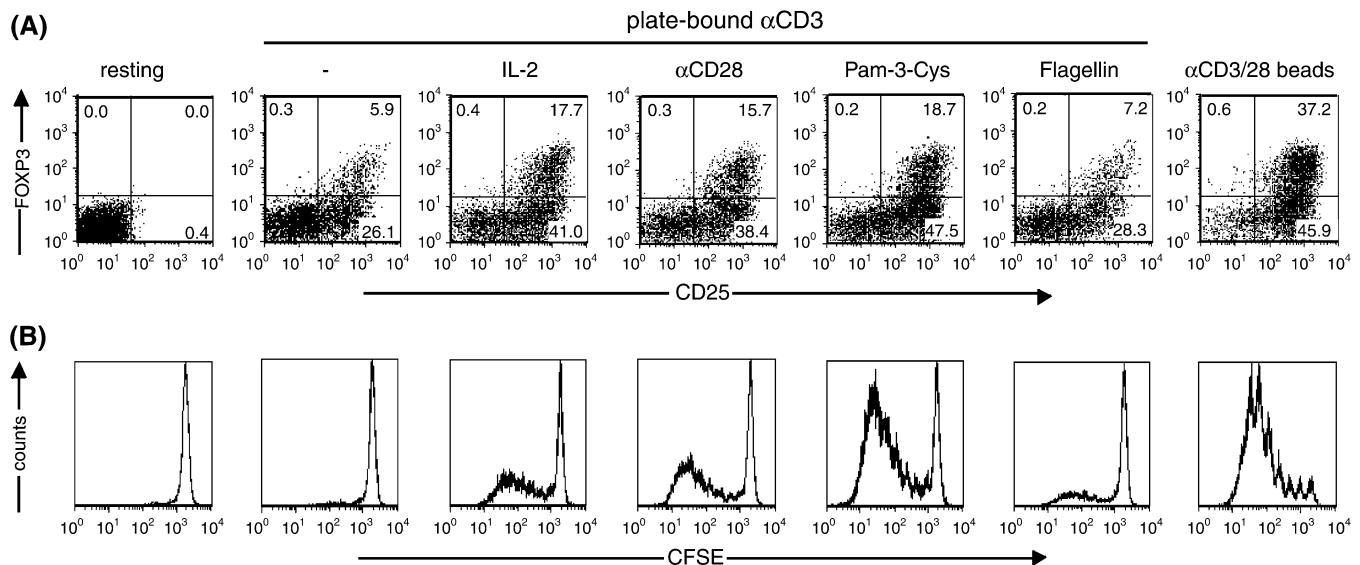
immobilized anti-CD3 alone elicited a small proportion of purified T cells to up-regulate FOXP3, and anti-CD28 co-stimulation enhanced this response.

#### Expression of FOXP3 in Teff cells does not negatively regulate cytokine production

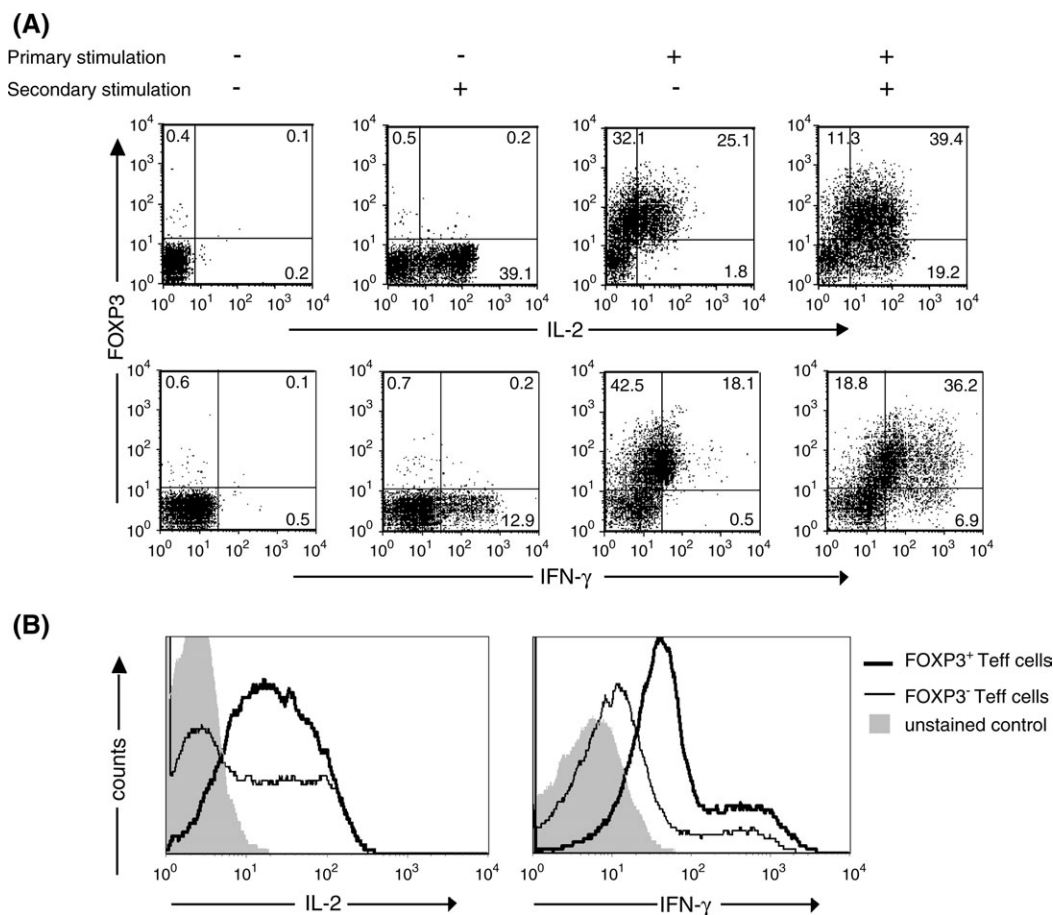
nTregs fail to produce significant amounts of most cytokines, likely due to the ability of FOXP3 to physically interact with and repress transcription from cytokine promoters (7, 17, 24, 25). To investigate the possibility that activation-induced FOXP3 might negatively regulate cytokine production in Teff cells, we determined whether induction of FOXP3 was correlated with suppression of IL-2 and IFN- $\gamma$ . Teff cells were stimulated with anti-CD3/CD28-coated beads for 72 h and then either directly analyzed, or re-stimulated with PMA and Ca<sup>2+</sup> ionophore to induce maximal cytokine production, before analysis of their capacity to produce IL-2 and IFN- $\gamma$ . As shown in Fig. 4, irrespective of whether the cells were re-stimulated, the FOXP3<sup>+</sup> Teff cells were the primary producers of IL-2 and IFN- $\gamma$ . As expected, nTregs analyzed in parallel produced very low amounts of IL-2 and IFN- $\gamma$  (data not shown). Thus expression of FOXP3 in Teff cells does not repress production of IL-2 or IFN- $\gamma$ .

#### Activation-induced FOXP3 does not suppress CD127 expression

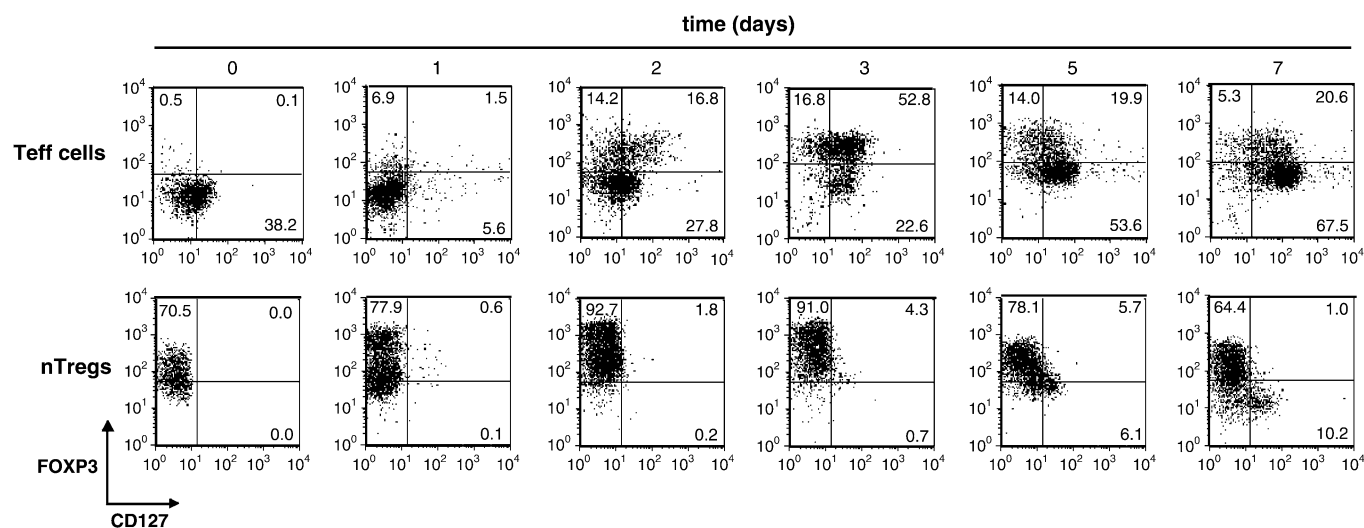
Recently, it was reported that human nTregs can be distinguished from Teff cells on the basis of low CD127 (IL-7R $\alpha$ ) expression (26, 27), possibly due to the capacity of FOXP3 to interact with and repress the CD127 promoter (26). In line with these reports, we found that *ex vivo* nTregs expressed low levels of CD127, and levels remained low over the course of activation (Fig. 5). Since sorting on the basis of CD25 does not result in a homogeneous population



**Fig. 3.** Co-stimulatory requirements for activation-induced FOXP3 expression. *Ex vivo* Teff cells were activated for 72 h in the presence of 10  $\mu$ g ml<sup>-1</sup> plate-bound anti-CD3 with or without co-stimulatory molecules (IL-2 100U ml<sup>-1</sup>, anti-CD28 1  $\mu$ g ml<sup>-1</sup>, Pam-3-Cys 1  $\mu$ g ml<sup>-1</sup> and flagellin 100 ng ml<sup>-1</sup>), or with anti-CD3/CD28-coated beads. (A) Expression of CD25 and FOXP3 (using 236A/E7, conjugated to PE) was determined after 72 h. (B) Extent of proliferation was determined by CFSE dilution after 5 days. Results are representative of three experiments.



**Fig. 4.** FOXP3<sup>+</sup> Teff cells produce IL-2 and IFN- $\gamma$ . (A) *Ex vivo* Teff cells were stimulated with anti-CD3/CD28-coated beads for 72 h prior to a secondary 4-h stimulation with PMA and Ca<sup>2+</sup> ionophore. Cells were then stained for IL-2, IFN- $\gamma$  and FOXP3 (using 236A/E7, conjugated to APC). Unstimulated cells were analyzed in parallel as a control. (B) *Ex vivo* Teff cells were exposed to primary and secondary stimulation as in (A) and histogram plots were overlaid by gating on the FOXP3<sup>+</sup> or FOXP3<sup>-</sup> fractions of Teff cells. Data are representative of four experiments.



**Fig. 5.** Activated Teff cells can co-express FOXP3 and CD127. Purified Teff cells and nTregs were activated with anti-CD3/CD28-coated beads and analyzed for expression of FOXP3 (using biotin-linked PCH101 and streptavidin-APC) and CD127 at the indicated times. Plots are representative of four experiments.

of nTregs (28), both FOXP3<sup>hi</sup> and FOXP3<sup>int</sup> cells were observed as early as 24 h and at day 7, a small percentage of FOXP3<sup>low</sup>CD127<sup>+</sup> T cells was detectable. In contrast to nTregs, expression of CD127 declined immediately after activation of Teff cells, and then reappeared in both FOXP3<sup>+</sup> and FOXP3<sup>-</sup> fractions: 33–83% of FOXP3<sup>+</sup> Teff cells co-expressed CD127 ( $n = 5$ ). These data indicate that activation-induced expression of FOXP3 in Teff cells is not sufficient to uniformly suppress CD127 expression, while high and constitutive FOXP3 expression in nTregs is correlated with their characteristic CD127<sup>low/-</sup> phenotype. Also of note is the observation that a proportion of early activated Teff cells are FOXP3<sup>+</sup>, CD127<sup>low/-</sup> and CD25<sup>+</sup>, and would not be distinguishable from nTregs on the basis of low CD127 expression. Thus, a FACS-sorting strategy based on isolation of CD127<sup>low/-</sup> cells may not be an infallible method for obtaining populations of pure nTregs constitutively expressing FOXP3, particularly from patients with infection or inflammation (29).

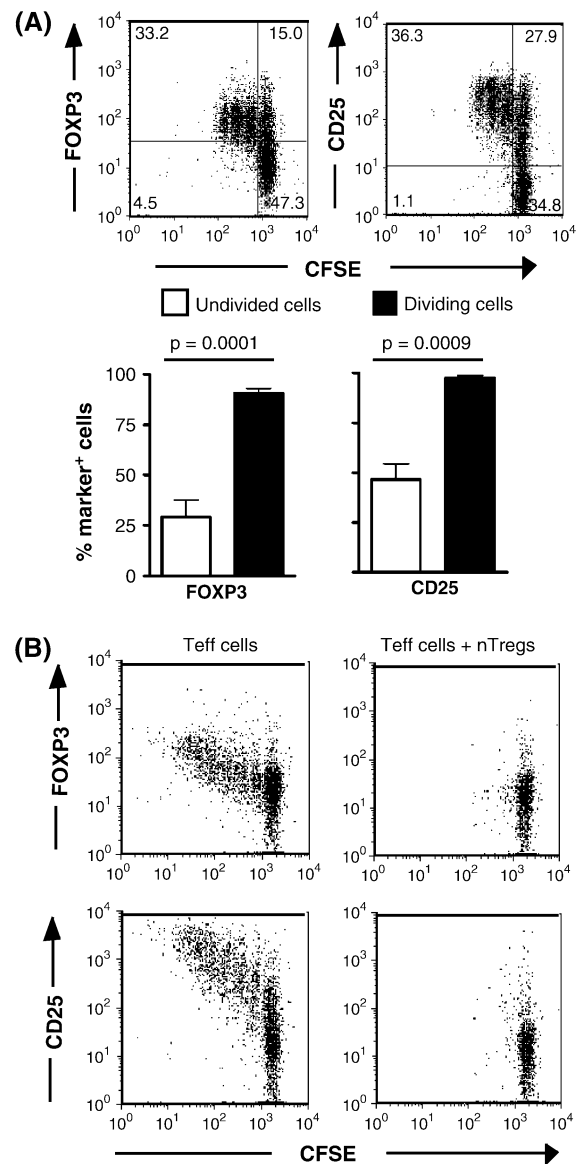
#### Activation-induced FOXP3 expression is up-regulated in dividing but not suppressed cells

nTregs are anergic *in vitro*, and we and others have demonstrated that enforced expression of FOXP3 in human CD4<sup>+</sup> T cells induces a hypo-responsive phenotype (7–9). We therefore hypothesized that activation-induced expression of FOXP3 in Teff cells could serve as a negative regulator of T cell activation and proliferation. To address this question, we labeled *ex vivo* Teff cells with CFSE and activated them with anti-CD3/CD28-coated beads. Interestingly, all proliferating cells up-regulated FOXP3, whereas undivided cells comprised a mixture of FOXP3<sup>+</sup> and FOXP3<sup>-</sup> cells (Fig. 6A). A similar pattern was observed when expression of CD25 was analyzed. Thus, activation-induced FOXP3 does not prevent progression through the cell cycle and, like CD25, is elevated in all dividing cells. These data also argue against the possibility that the FOXP3<sup>+</sup> cells had converted to Tregs since the latter would be expected to be hypo-responsive in the absence of exogenous IL-2.

We further hypothesized that if induction of FOXP3 is a normal consequence of T cell activation, nTregs should suppress its expression in Teff cells. Alternatively, co-culture with nTregs could amplify the levels of FOXP3 in suppressed cells as a mechanism of infectious tolerance (30, 31). To investigate these possibilities, Teff cells were activated in the absence or presence of nTregs (Fig. 6B). Activated Teff cells consistently up-regulated FOXP3 and CD25 whereas in co-cultures with nTregs, both proliferation and expression of these markers were clearly suppressed. Thus, the anergic state of suppressed Teff cells is not due to induced expression of FOXP3.

#### Activation-induced expression of FOXP3 in Teff cell lines

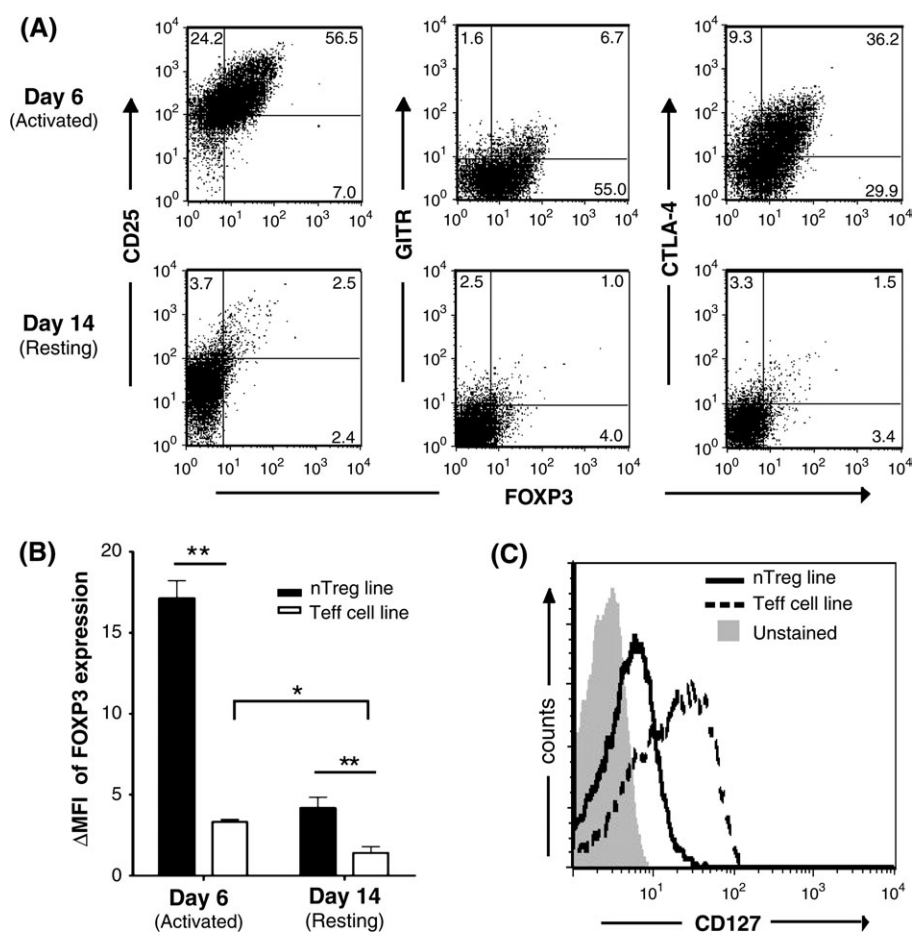
From the experiments described above, it appeared that FOXP3 behaved as a classical activation marker in Teff cells, with peak expression levels occurring at the time of maximal activation, and then gradually subsiding upon entry into the resting phase. In order to determine whether this pattern of FOXP3 expression recurred during repeated cycles of



**Fig. 6.** All proliferating Teff cells express FOXP3. (A) *Ex vivo* Teff cells were CFSE labeled and levels of CFSE, CD25 and FOXP3 were analyzed at 72 h. FACS plots are a representative of six experiments, which are averaged in graphs below. (B) *Ex vivo* Teff cells were CFSE labeled and stimulated with APCs and anti-CD3 in the presence or absence of nTregs at a 1:1 ratio. FOXP3 and CD25 expression was analyzed in CFSE<sup>+</sup> populations after 96 h. FOXP3 staining was carried out using biotin-linked PCH101 and streptavidin-APC. Data are representative of three experiments.

activation, we monitored its expression in parallel with other nTreg markers in Teff cell lines. As shown in Fig. 7 (A and B), a significant proportion of cells in the activated Teff cell lines transiently expressed FOXP3 ( $45.6 \pm 16.3\%$ ,  $n = 3$ ) along with CD25, CTLA-4 and GITR. Similar to our findings with *ex vivo* cells, the intensity of FOXP3 expression and of other nTreg-associated markers (data not shown) in Teff cell lines was significantly lower than in nTreg lines.

In view of the recent finding that low expression of CD127 may be a more specific cell-surface marker for nTregs than CD25, we also investigated whether Teff cell and nTreg lines



**Fig. 7.** Kinetics of expression of nTreg-associated markers in Teff cell lines. (A) Teff cell lines were analyzed at day 6 (activated) and day 14 (resting) after activation for the indicated markers. (B) The average intensity of FOXP3 expression (determined using either PE- or biotin-linked PCH101 and streptavidin-APC) was compared between Teff cell and nTreg lines after normalizing to unstained controls for each sample. Statistical significance: \* $P < 0.05$  and \*\* $P < 0.01$ . (C) The expression of CD127 was determined in resting nTreg and Teff cell lines. Data are representative of three independent cell lines from different donors.

remained CD127<sup>+</sup> and CD127<sup>low</sup>, respectively. Upon analysis in the resting phase, nTreg lines displayed significantly lower expression of CD127 (5.1-fold  $\pm$  3.1-fold lower MFI,  $P = 0.036$ ,  $n = 3$ ) than did Teff cell lines, suggesting that the transient FOXP3 expression that occurs in activated Teff cells is not sufficient to repress their CD127 expression in the long term (Fig. 7C).

## Discussion

In this report, we investigated the kinetics and expression profile of activation-induced FOXP3 in human Teff cells, and determined its effect on proliferation and cytokine production. We found that in humans, all dividing Teff cells have the potential to become FOXP3 positive, independently of CD28 co-stimulation, and conclude that expression of FOXP3 is a normal consequence of CD4<sup>+</sup> T cell activation. Consequently, simple analysis of FOXP3 expression can no longer be used as a surrogate marker of nTregs in humans (12, 20, 21). Rather, like CD25, FOXP3 appears to be strictly associated with the unique phenotype and function of nTregs only when it is expressed constitutively and at high

levels. In addition, evidence that expression of FOXP3 is repressed in suppressed Teff cells, indicates that nTregs do not induce anergy or mediate infectious tolerance via induction of this transcription factor. The role of FOXP3 in nTregs appears to be related to its ability to repress a number of different target genes, particularly cytokines (17, 24–26). In order to determine whether activation-induced FOXP3 can repress known transcriptional targets, we investigated the expression of several of these molecules in Teff cells. In contrast to nTregs, we found that FOXP3<sup>+</sup> Teff cells produced significantly more IL-2 and IFN- $\gamma$  compared with FOXP3<sup>-</sup> cells in the same culture. Together with our observations that activation-induced FOXP3 was not sufficient to suppress CD127 expression, and that FOXP3<sup>+</sup> cells were highly proliferative, these data strongly suggest that the function of FOXP3 in Teff and nTregs may not be equivalent. A differential function may be related to the insufficient and/or unsustained nature of FOXP3 expression in Teff cells. Highly activated Teff cells may also have an imbalance in additional proteins required for suppression of these genes, as FOXP3 exerts its repressor activity in concert with several other co-factors (32).



Some molecules up-regulated upon TCR stimulation, such as CD25 and GITR, serve to enhance T cell activation by increasing sensitivity to cytokines or chemokines, or providing co-stimulatory signals (33, 34). In contrast, others, such as CTLA-4 serve to curb T cell activation and dampen the immune response by initiating inhibitory signals within or between cells (35). The kinetics of FOXP3 expression in Teff cells closely resemble those of CTLA-4, as both of these markers are induced later than CD25 and CD69. Interestingly, it has recently been reported that CTLA-4 co-stimulation is required for induction of transforming growth factor- $\beta$  (TGF- $\beta$ )-mediated Foxp3 expression in mouse cells (36). It is therefore possible that CTLA-4 ligation in activated human Teff cell populations contributes to activation-induced FOXP3 expression, and that FOXP3 and CTLA-4 act as co-operative arms in a negative feedback loop that limits T cell expansion.

It has been previously reported that induction of FOXP3 in Teff cells is associated with the development of Tregs and suppression (10, 11). Our data support the conclusion that the majority of Teff cells that express FOXP3 at the peak of activation do not permanently convert to Tregs. We attempted to directly test whether FOXP3<sup>+</sup> Teff cells acquired suppressive capacity, but found that when FOXP3 was maximally expressed (3 days after activation), these experiments were not feasible due to induction of cell death upon reactivation and IL-2 consumption in co-cultures (data not shown). Indirect evidence to support the conclusion that FOXP3<sup>+</sup> Teff cells are not Tregs includes the findings that activation-induced expression of FOXP3 did not suppress cytokine production, CD127 expression or proliferation. We can speculate, however, that given the appropriate culture conditions, a small subset of these cells may not down-regulate FOXP3 and therefore ultimately develop into functional Tregs. A number of recent reports have described tolerance-promoting agents, such as TGF- $\beta$ , that can induce suppressor cells from murine or human Teff cells (37). These exogenous factors may act via enhancing and/or prolonging activation-induced FOXP3 expression, or alternatively, by triggering the assembly of other molecular complexes necessary for suppressor function. Maintenance of sustained FOXP3 expression as T cell activation wanes may be key for acquisition of suppressor function and development of peripherally induced Tregs.

The role of FOXP3 in Teff cells remains a major outstanding question. Over-expression and molecular studies have clearly shown that viral long terminal repeat-driven expression of FOXP3 in human Teff cells limits their ability to proliferate and produce IL-2 and IFN- $\gamma$  (7–9), suggesting it may have a negative regulatory role. Our data, together with recent evidence from Gavin *et al.* (12), suggest, in contrast, that the relatively low levels and transient nature of endogenous FOXP3 expression in activated Teff cells are not sufficient to engender these effects. On the other hand, FOXP3 is also involved in transcriptional activation of genes, and its ability to up-regulate CD25 may in fact act to enhance Teff cell activation (24). Thus, it is possible that activation-induced FOXP3 expression may fine-tune negative and/or positive aspects of Teff cell proliferation and activation.

Our data from IPEX patients, who, regardless of the site of the FOXP3 mutation, display a defect in the capacity of Teff

cells to produce cytokines, further support the concept that the role of FOXP3 is not limited to nTregs (6). In striking contrast to IPEX patients, Teff cells from mice with mutations in Foxp3 produce higher amounts of inflammatory cytokines than Teff cells from their wild-type littermates (38, 39), indicating this alternate role is likely a species-specific phenomenon. Recent data indicating that FOXP3 prevents the interaction between NFAT and AP1, and co-operates with NFAT to switch T cells into nTregs (24) suggest that the differential role of FOXP3 in Teff cells versus nTregs could be related to differential activity/expression of NFAT and/or AP1. Overall, the concept that the exclusive role of FOXP3 is to act as a molecular switch for the development of nTregs needs to be re-examined and further studies are required to elucidate whether FOXP3 has a positive and/or negative regulatory role in the context of CD4<sup>+</sup> T cell activation.

### Acknowledgements

We gratefully acknowledge the support of Barrett Benny, Michael Barnett and Raewyn Broady at the Cell Separator Unit at Vancouver General Hospital, for providing PBMCs. The anti-FOXP3 antibody used for western blotting was generously provided by Steven Ziegler (Benaroya Research Centre, Seattle, WA, USA). We thank Paul Orban for critical reading of the manuscript. This work was supported by grants from the Canadian Institutes for Health Research (CIHR) (MOP127506, to M.K.L.) and the Italian Telethon Foundation Rome (HSR-TIGET-New Exploratory Project and GGP 04285 to R.B. and M.G.R.). M.K.L. holds a Canada Research Chair in Transplantation and is a Michael Smith Foundation for Health Research (MSFHR) Scholar. S.E.A. holds a MSFHR Junior Graduate Studentship award and a CIHR Canada Graduate Scholarship Doctoral Award. S.Q.C. holds a CIHR-MSFHR Transplantation training program award. N.K.C. holds a MSFHR Senior Graduate Studentship award. T.S.S. holds a CIHR New Investigator Award.

### Abbreviations

APC	antigen-presenting cell
CFSE	5- (and 6-) carboxyfluorescein diacetate succinimidyl ester
CIHR	canadian Institutes for Health Research
CTLA-4	cytotoxic T-lymphocyte-associated protein4
FoxP3	forkhead box P3
GITR	glucocorticoid-induced tumor necrosis factor receptor
IPEX	immune dysregulation polyendocrinopathy, enteropathy X-linked syndrome
MFI	mean fluorescence intensity
MSFHR	Michael Smith Foundation for Health Research
nTreg	naturally occurring CD4 <sup>+</sup> CD25 <sup>+</sup> T regulatory cell
Teff cell	T effector cell
TGF- $\beta$	transforming growth factor- $\beta$
PMA	phorbol myristate acetate

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