

STAT5-signaling cytokines regulate the expression of FOXP3 in CD4⁺CD25⁺ regulatory T cells and CD4⁺CD25[−] effector T cells

Laura Passerini¹, Sarah E. Allan², Manuela Battaglia¹, Sara Di Nunzio¹, Alicia N. Alstad², Megan K. Levings², Maria G. Roncarolo^{1,3} and Rosa Bacchetta¹

¹San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Via Olgettina 58, 20132 Milan, Italy

²Department of Surgery, University of British Columbia and Immunity and Infection Research Centre, Vancouver Coastal Health Research Institute, British Columbia V6H 3Z6, Canada

³Vita-Salute San Raffaele University, Milan, Italy

Keywords: γ -chain signaling cytokines, suppressive activity, transcription factor

Abstract

Forkhead box P3 (FOXP3) is considered a specific marker for CD4⁺CD25⁺ regulatory T (Treg) cells, but increasing evidence suggests that human CD4⁺CD25[−] effector T (Teff) cells can transiently express FOXP3 upon activation. We demonstrate that the signal transducer and activator of transcription 5 (STAT5)-signaling cytokines, IL-2, IL-15 and to a lesser extent IL-7, induce FOXP3 up-regulation *in vitro* in activated human Teff cells. In contrast, cytokines which do not activate STAT5, such as IL-4 or transforming growth factor- β alone, do not directly induce FOXP3 expression in activated Teff cells. Moreover, expression of a constitutively active form of STAT5a is sufficient to induce FOXP3 expression in Teff cells. Expression of FOXP3 in activated Teff cells requires both TCR-mediated activation and endogenous IL-2, but is not dependent on cell division and does not induce suppressive function. The presence of STAT5-activating cytokines is also required to maintain high FOXP3 expression and suppressive activity of Treg cells *in vitro*. These data indicate that activation of STAT5 sustains FOXP3 expression in both Treg and Teff cells and contribute to our understanding of how cytokines affect the expression of FOXP3.

Introduction

Although IL-2 was first identified as potent T cell growth factor (1), it has more recently been demonstrated that a major function of IL-2 is the maintenance of peripheral T cell tolerance (2, 3 and reviewed in 4). *IL2*^{−/−} (5) as well as IL-2R α (CD25)-deficient mice (6, 7) are characterized by hyperactivation of the CD4⁺ T cell compartment, which leads to a lethal multiorgan autoimmunity with anemia, auto-antibodies and severe colitis. IL-2-deficient mice, however, have normal numbers of regulatory T (Treg) cells (8) which are functional (9). Therefore, the precise role of IL-2 in tolerance and Treg cell biology remains to be defined. IL-2 signals through a receptor complex consisting of three subunits, namely the IL-2R α (CD25), IL-2R β (CD122) and common γ -chain (γ c) (CD132) (10). The high-affinity receptor is expressed by effector T (Teff) cells upon activation (11) and sustains their expansion and function in response to IL-2. In addition to IL-2, the only other cytokine known to signal through the IL-2R β – γ c receptor complex is IL-15 (12). The γ c is also shared

as signaling subunit of the receptors for IL-4, IL-7, IL-9, IL-15 and IL-21 (reviewed in 13).

The IL-2R α subunit is constitutively expressed at high level on the surface of naturally occurring CD4⁺CD25⁺ Treg cells (14), a distinct subset of T cells which has a key role in controlling and maintaining immune tolerance (15). Much evidence indicates that CD4⁺CD25⁺ Treg cells are able to suppress immune responses to auto- and allo-antigens, tumor antigens and infectious agents *in vivo* (reviewed in 16) and that they are dependent on exogenous IL-2 for their homeostasis and function (3, 4, 17). Suppression by CD4⁺CD25⁺ Treg cells is cell–cell contact dependent *in vitro* and requires signaling through IL-2 since both *in vitro* and *in vivo* suppressive activity of Treg cells is abrogated when IL-2 signaling is blocked (3, 18). Forkhead box P3 (FOXP3) has been identified as Treg cell lineage specification factor, in both murine and human T cells (19). Recently, it has been demonstrated that IL-2 up-regulates the expression of

FOXP3 in human CD4⁺CD25⁺ T cells, both *in vitro* and *in vivo* (20–22). However, the molecular mechanisms that link IL-2 to induction of FOXP3 expression and regulation of suppressive function remain unclear.

Importantly, expression of FOXP3 does not exclusively occur in CD4⁺CD25⁺ Treg cells since in humans it can also be transiently induced in activated CD4⁺CD25[−] Teff cells, which do not express FOXP3 in the resting state (23–27). The function of FOXP3 and the molecular factors that regulate its expression in Treg versus Teff cells remain to be defined.

In this study, we investigated the role of cytokines in regulating FOXP3 expression in human Teff and Treg cells. We found that expression of FOXP3 in CD4⁺CD25[−] Teff and CD4⁺CD25⁺ Treg cells is dependent on the presence of cytokines that activate signal transducer and activator of transcription 5 (STAT5), such as IL-2, IL-7 and IL-15. In contrast, IL-4 or transforming growth factor- β (TGF- β) alone was not sufficient to induce FOXP3 expression in activated CD4⁺CD25[−] Teff cells, in short-term *in vitro* culture. Furthermore, expression of a constitutively active form of STAT5a could induce expression of FOXP3 in Teff cells. These data indicate that activation of STAT5 is a critical factor in the induction and maintenance of FOXP3 expression in Teff and Treg cells, respectively.

Methods

Cell purification

Peripheral blood was obtained upon written informed consent from healthy donors in accordance with local ethical committee approval (Protocol TIGET03 and UBC CREB C03-0062). PBMCs were prepared over Ficoll–Hypaque gradients. CD4⁺ T cells were purified with the CD4⁺ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany), subsequently separated into CD25⁺ and CD25[−] fractions by positive selection (Miltenyi Biotec) and were 85–90% pure.

Short-term *in vitro* culture of T cells

Purified CD4⁺CD25⁺/CD25[−] T cells were cultured in X-VIVO15 medium (Cambrex, East Rutherford, NJ, USA) supplemented with 5% human serum (Cambrex) and 100 U ml^{−1} penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) for a period of 24–96 h with 10 μ g ml^{−1} of immobilized anti-CD3 (Janssen-Cilag, Raritan, NJ, USA) plus 1 μ g ml^{−1} of soluble anti-CD28 (BD Biosciences, San Jose, CA, USA) mAbs, with or without cytokines or in the presence of cytokines alone. At fixed time points, T cells were stained for FACS analysis or tested in functional assays. When evaluation of proliferative response was required, CD4⁺CD25[−] T cells were labeled with carboxyfluorescein diacetate succinimidylester (CFSE) (Invitrogen), as previously described (28) and stimuli/blockers were added 24 h after activation. Cells were cultured for additional 72 h and analyzed by FACS.

Cytokines and cytokine-signaling blockers

The following cytokines were added as indicated: IL-2 100 U ml^{−1} (Chiron, Novartis Vaccines and Diagnostics, Emeryville, CA, USA), IL-4 200 ng ml^{−1} (R&D Systems, Minneapolis, MN, USA), IL-7 10 ng ml^{−1} (Pepro Tech, Rocky Hill, NJ, USA), IL-15 10 ng ml^{−1} (R&D Systems) and TGF- β 1 1 ng

ml^{−1} (R&D Systems). When necessary, IL-2 signaling was blocked using 10 μ g ml^{−1} of anti-IL-2R α mAb clone 22722.2 (R&D Systems). In order to block STAT5-mediated signal transduction 2-(1,1-dimethyl)-9-fluoro-3,6-dihydro-7H-benz[h]-imidaz[4,5-f]isoquinolin-7-one (Jak Inhibitor I in Solution) (Calbiochem, Merck KGaA, Darmstadt, Germany) was used at a concentration of 1 μ M; this compound displays inhibitory activity against Jak1, Jak3 and Tyk2 and blocks the phosphorylation of STAT5 (29). Rapamycin (Sigma, St Louis, MO, USA) 100 nM (30) was used to block the phosphatidylinositol-3-kinase (PI3K) pathway.

FACS analysis

T cells were stained with anti-CD4 and anti-CD25 mAbs (BD Biosciences) FITC or PE coupled, respectively. Expression of FOXP3 was determined by staining with allophycocyanin-conjugated anti-FOXP3 mAb (clone PCH101 or clone 236A/E7, eBioscience San Diego, CA, USA), following the manufacturer's instructions. The specificity of the FOXP3 staining was confirmed by comparing results obtained with clone PCH101 with those obtained with clone 259D (Biolegend, San Diego, CA, USA) (data not shown). Staining for CD69, HLA-DR and CD40L (BD Biosciences) was performed according to the manufacturer's instructions.

Suppression assay

CD4⁺CD25[−] T cells, used as responder cells, were plated in 96-well round-bottom plates (Corning, Corning, NY, USA) at a density of 50 000 or 100 000 cells per well, for [³H]-thymidine incorporation and CFSE dilution assay, respectively, in a final volume of 200 μ l of complete medium and activated in the presence of CD3-depleted PBMC (irradiated 6000 rad) and soluble anti-CD3 mAb (1 μ g ml^{−1}). Suppressor cells were added at a ratio of 1:1. When CFSE dilution was used as read out, cells were analyzed by FACS after 4 days. Staining of responder cells and calculation of the proportion of CFSE⁺ cells proliferating *in vitro* was performed as described previously (28). When [³H]-thymidine incorporation was used as a read out, cells were pulsed for 16 h with 1 μ Ci per well of [³H]-thymidine (Amersham Biosciences, Uppsala, Sweden) after 72 h. Cells were harvested and counted in a scintillation counter.

Lentiviral production and transduction of CD4⁺CD25[−] T cells

A cDNA encoding murine signal transducer and activator of transcription 5a (STAT5a) containing two point mutations which render it constitutively active (STAT5a-CA) (31) was kindly provided by A. Mui and T. Kitamura. STAT5a-CA was cloned into a bidirectional lentiviral vector encoding Δ NGFR as a marker gene (32). Lentivirus was produced by transient 4-plasmid transfection of HEK 293T cells as previously described (33). For T cell transduction, CD4⁺CD25[−] T cells were activated overnight with soluble anti-CD3 mAb (1 μ g ml^{−1} OKT3), rIL-2 (100 U ml^{−1}) and autologous irradiated antigen-presenting cells (APCs) at a 1:5 ratio of T cells to APCs. T cells were infected with lentiviral supernatant at a multiplicity of infection of 10. Δ NGFR⁺-transduced T cells were purified 8 days following transduction using Δ NGFR-select beads (Miltenyi Biotec) and expanded in IL-2-containing

medium. Untransduced cells tested in parallel were equivalent to control-transduced cells in all aspects of phenotype and function (data not shown).

Statistical analysis

Results are presented as mean $\pm$ standard error (SE). Analyses were performed either with two-tailed paired or unpaired Student's *t*-test as indicated in the text. *P* values <0.05 were considered significant.

Results

Activation-induced FOXP3 expression in CD4⁺CD25⁺ T cells is dependent on STAT5-signaling cytokines

It has recently been reported that in humans, FOXP3 is not only expressed in Treg cells but also in Teff cells (24, 27, 34).

To better understand the mechanistic basis of FOXP3 induction in Teff cells, we studied the kinetics of FOXP3 expression upon activation with anti-CD3 and anti-CD28 mAbs in human CD4⁺CD25⁺ Teff cells. Activation-induced FOXP3 expression peaked after 72 h with an average of $7.9 \pm 0.8\%$ CD4⁺FOXP3⁺ T cells (mean $\pm$ SE, *n* = 19) (Fig. 1A and Table 1). We recently reported similar results in a separate study, although the percentage of FOXP3⁺ cells was higher, due to the use of stronger activation stimuli (27). Addition of exogenous IL-2 significantly increased the percentage of FOXP3⁺ Teff cells (Fig. 1A and Table 1). Furthermore, in the presence of IL-2, expression of FOXP3 persisted for up to 7 days (data not shown). Conversely, FOXP3 expression was not induced if the cells were activated in the presence of a neutralizing anti-IL-2R α mAb (Fig. 1A and Table 1).

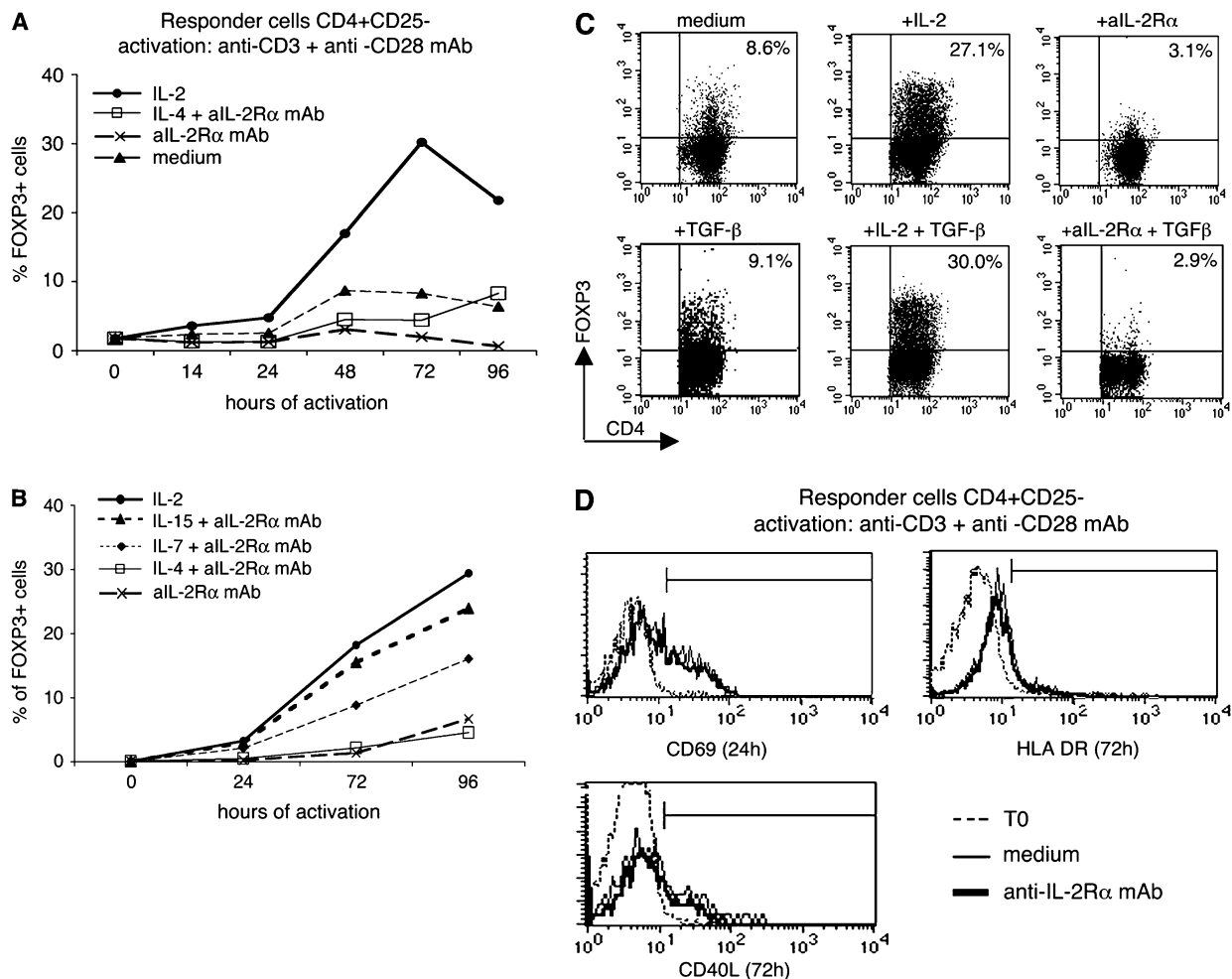


Fig. 1. FOXP3 up-regulation upon activation of CD4⁺CD25⁺ T cells is dependent on STAT5-signaling cytokines. (A) CD4⁺CD25⁺ T cells were activated in the presence of plate-bound anti-CD3, soluble anti-CD28 mAbs either alone or in combination with IL-2 or anti-IL-2R α mAb with or without IL-4. (B) Alternatively, Teff cells were activated in the presence of anti-CD3 and anti-CD28 mAbs and IL-2 or anti-IL-2R α mAb plus IL-4, IL-7 or IL-15. After 14–96 h of incubation, cells were stained to determine FOXP3 expression. Percentages of FOXP3⁺ T cells in the CD4⁺ gate measured at each time point are plotted in the graphs (one representative donor). (C) Teff cells were activated in the presence of anti-CD3 and anti-CD28 mAbs and IL-2 or TGF- β plus or minus either IL-2 or anti-IL-2R α mAb. FOXP3 expression was determined after 72 h of culture (one representative donor). (D) The expression of the T cell activation markers CD69 (left panel), HLA-DR (middle panel) and CD40L (right panel) was evaluated in CD4⁺CD25⁺ Teff cells activated with anti-CD3 plus anti-CD28 mAbs in the presence (thick line) or in the absence (thin line) of anti-IL-2R α mAb. Staining at T0 (dashed line) is also shown. Region of positive staining was set based on the isotype control and is marked in the histograms (representative of three donors for CD69 and CD40L and four for HLA-DR).

In order to determine whether other T cell growth factors could replace IL-2, we first tested whether IL-4, which also

Table 1. Percentage of FOXP3⁺ cells in activated^a CD4⁺CD25⁻ T cells

Culture conditions	FOXP3 expression ^b (mean % ± SE)	n	P ^c versus IL-4 + aIL-2Rα
Medium	7.9 ± 0.8	19	<0.005
IL-2	20.3 ± 1.9	19	<0.0001
IL-4	8.9 ± 1.6	6	<0.005
IL-4 + aIL-2Rα	3.8 ± 0.5	10	
aIL-2Rα	2.6 ± 0.4	15	NS
IL-7 + aIL-2Rα	13.1 ± 1.4	7	<0.0001
IL-15 + aIL-2Rα	18.2 ± 2.7	7	<0.0001
TGF-β + aIL-2Rα	3.5 ± 0.8	11	NS
TGF-β	8.0 ± 1.1	11	<0.005
TGF-β + IL-2	22.6 ± 3.9	5	<0.0001

aIL-2Rα, anti-IL-2Rα mAb; NS, not significant.

^aActivation: anti-CD3 + anti-CD28 mAbs.

^bValues refer to 72 h cultures.

^cStatistical analysis was performed using the unpaired Student's *t*-test.

signals through the common γc, could also enhance expression of FOXP3 in Teff cells. In the presence of IL-4, FOXP3 was expressed at levels comparable to those in Teff cells activated in medium alone (Table 1). Furthermore, when production of endogenous IL-2 was neutralized by IL-2R blockade, addition of IL-4 did not result in a significant increase in FOXP3 expression. Only a small proportion of cells with low FOXP3 expression was detected at the latest time point tested (i.e. 96 h after activation), probably due to reduced efficacy of the anti-IL-2Rα-blocking mAb after 4 days of culture (Fig. 1A).

Like IL-2, T cell stimulation with IL-7 or IL-15 leads to the phosphorylation and subsequent translocation of STAT5 to the nucleus (35, 36). In contrast, IL-4, which also signals via the common γc, activates STAT6 (37). The inability of IL-4 to induce STAT5 phosphorylation in CD4⁺ T cells was confirmed by FACS analysis (data not shown). We therefore investigated whether, like IL-2, IL-7 and IL-15 could induce FOXP3 expression in activated CD4⁺CD25⁻ Teff cells. Both cytokines were able to induce FOXP3 expression in activated Teff cells, even when the IL-2R was blocked. In the

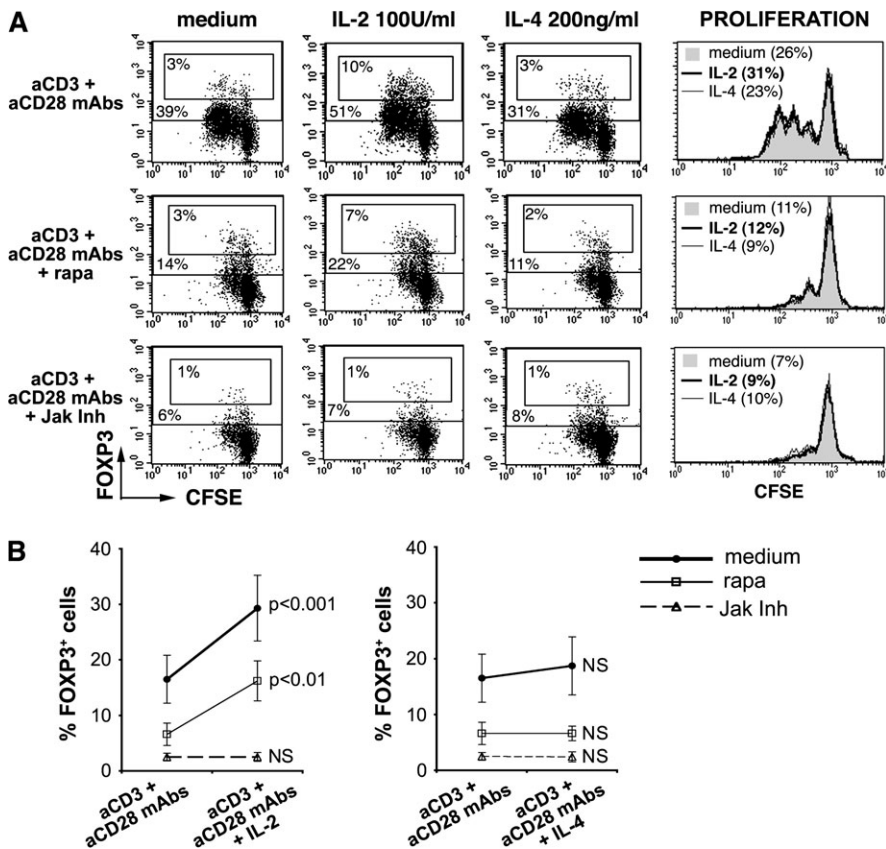


Fig. 2. FOXP3 expression and proliferation. CFSE-labeled CD4⁺CD25⁻ Teff cells were activated in the presence of anti-CD3 plus anti-CD28 mAbs. After 24 h of activation, alternatively IL-2 or IL-4, alone or in combination with either rapamycin (rapa) or a Jak inhibitor (Jak Inh), was added to the culture medium. After 72 h, the cells were stained to determine FOXP3 expression. Region of positive staining was set based on the isotype control. (A) Percentages of FOXP3⁺ (large frame) and FOXP3^{bright} (small frame) cells are reported in the plots. Histograms show CFSE dilution of untreated (gray peaks) or IL-2 (bold line)/IL-4 (thin line)-treated samples either alone (upper panels) or in the presence of rapamycin (middle panels)/Jak inhibitor (lower panels). Numbers in the histogram plots indicate percent of proliferating precursors (representative of six donors). (B) Mean % FOXP3⁺ cells ± SE of *n* = 6 donors after anti-CD3 + anti-CD28 mAbs ± IL-2/IL-4 treatment in the presence or absence of either rapamycin (rapa) or Jak inhibitor (Jak Inh) is plotted in the graphs. Statistical analysis was performed using the paired Student's *t*-test; *P* values versus anti-CD3 + anti-CD28 mAbs treatment is reported in the graphs. NS, not significant.

presence of IL-15, FOXP3 was expressed at levels comparable to those observed in the presence of IL-2. Although IL-7 could induce FOXP3, it was less efficient than IL-2 or IL-15 (Fig. 1B and Table 1). The percentage of FOXP3⁺ T cells in the presence of IL-4 was significantly lower when compared with T cells activated in medium alone or in the presence of IL-2, IL-7 or IL-15 ($P < 0.05$ versus IL-4 + anti-IL-2R α mAb) (Fig. 1B and Table 1). Addition of these cytokines without TCR activation did not induce FOXP3 up-regulation in CD4⁺CD25[−] Teff cells (data not shown).

TGF- β , which does not activate STAT5, has been shown to induce FOXP3 expression in murine (38, 39) and human (40, 41) activated CD4⁺CD25[−] T cells. We observed that exogenous TGF- β did not enhance FOXP3 expression in activated human CD4⁺CD25[−] T cells in short-term culture, when signaling through the IL-2R was blocked (Fig. 1C). Even in the absence of anti-IL-2R α mAb, addition of TGF- β did not result in a significant increase in FOXP3 expression compared with cells activated in medium alone for 72 h (Fig. 1C and Table 1). Moreover, addition of both TGF- β and IL-2 did not result in a significant increase in FOXP3 compared with expression in the presence of exogenous IL-2 alone (Fig. 1C and Table 1). However, after 7 days of culture, addition of TGF- β did result in a small increase in FOXP3 expression (range 4–14%, $n = 3$) compared with IL-2 alone (data not shown).

In order to exclude the possibility that the lack of FOXP3 expression in the presence of anti-IL-2R α mAb was due to impaired T cell activation, we monitored the expression of other activation-induced markers, namely CD69, HLA-DR and CD40L, in CD4⁺CD25[−] Teff cells after anti-CD3 and anti-CD28 mAbs stimulation in the presence or absence of anti-IL-2R α mAb (Fig. 1D). No difference in the expression of any of these activation markers in CD4⁺CD25[−] Teff cells cultured with or without anti-IL-2R α mAb was observed.

Treatment of CD4⁺CD25[−] Teff cells with a Jak inhibitor prevents activation-induced FOXP3 expression

We have previously shown that expression of FOXP3 in Teff cells correlates with proliferation (27). In order to address whether cytokine-induced FOXP3 expression in activated Teff cells was dependent on proliferation, IL-2 signaling or

on both of these events, Teff cells were stimulated in the presence of specific inhibitors. A Jak inhibitor was used to block both proliferation and STAT5 activation, and rapamycin was used to inhibit proliferation (via blockade of the PI3K pathway) without affecting activation of STAT5. Although the overall proportion of FOXP3⁺ cells was lower, in the presence of rapamycin, IL-2 retained its capacity to induce expression of FOXP3 ($P < 0.01$ versus anti-CD3 + anti-CD28 mAbs + rapamycin) (Fig. 2A and B). In contrast, blockade of Jak activation inhibited the capacity of IL-2 to increase FOXP3 expression ($P > 0.05$ versus anti-CD3 + anti-CD28 mAbs + Jak inhibitor) (Fig. 2B). These data indicate that IL-2-induced activation of the Jak/STAT5 pathway is required for FOXP3 induction, independently of proliferation.

Ectopic expression of constitutively active STAT5 induces FOXP3 expression but not regulatory function in CD4⁺CD25[−] T cells

In order to directly demonstrate that activation of STAT5 can induce expression of FOXP3 in Teff cells, we investigated the effects of over-expressing a constitutively active form of STAT5a (STAT5a-CA) (31) in CD4⁺CD25[−] Teff cells. The cDNA was expressed by a bidirectional lentiviral vector, which also encodes Δ NGFR as a cell-surface marker for tracking and sorting of transduced cells (32). CD4⁺CD25[−] T cells were transduced with empty vector (expressing Δ NGFR only) or mSTAT5a-CA lentivirus, purified on the basis of Δ NGFR expression and tested for FOXP3 expression. Expression of STAT5a-CA resulted in a marked up-regulation in FOXP3 expression compared with control-transduced cells: in the resting state, the percentage of FOXP3⁺ cells in STAT5a-CA-expressing cells was $38.4 \pm 6.6\%$ greater than control-transduced cells (Fig. 3A). The mean fluorescence intensity (MFI) of FOXP3 expression was 3.8 ± 1.2 -fold higher in the STAT5a-CA-expressing cells. CD25 was also elevated in STAT5a-CA-transduced T cells (Fig. 3A), consistent with the known ability of STAT5 to directly induce expression of this gene (42, 43). These data strongly suggest that signaling through STAT5 causes a significant and sustained increase of FOXP3 expression in Teff cells.

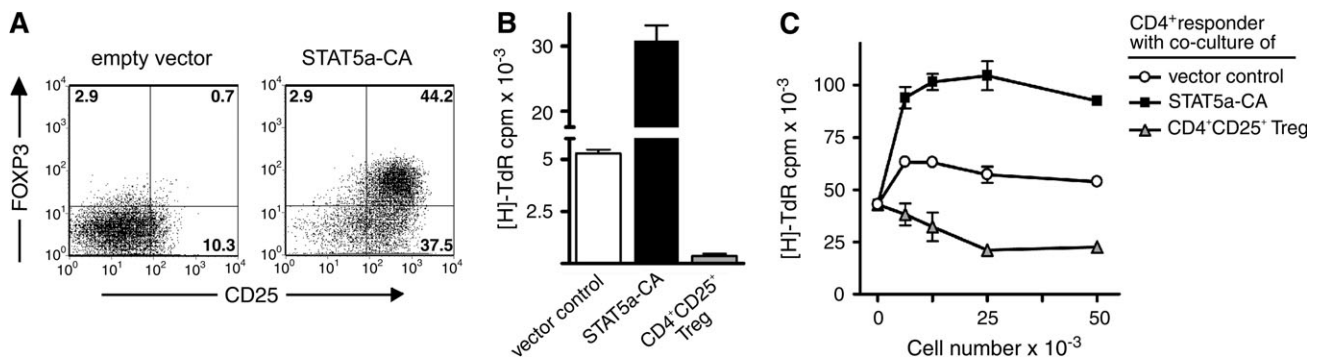


Fig. 3. Constitutive STAT5a signaling induces FOXP3 expression but not regulatory function in CD4⁺CD25[−] T cells. CD4⁺CD25[−] T cells were activated and transduced with lentiviral vectors, purified for marker gene expression and assayed in the resting state. (A) CD25 and FOXP3 (clone 236A/E7) expressions were tested by flow cytometry. (B) Proliferation of transduced T cells in response to allo-APCs and anti-CD3 mAb. (C) Suppressive capacity of transduced T cells was determined. In (B and C) expanded CD4⁺CD25⁺ Treg cells are shown as a positive control. Data are representative of experiments performed with the T cells of three different normal donors.

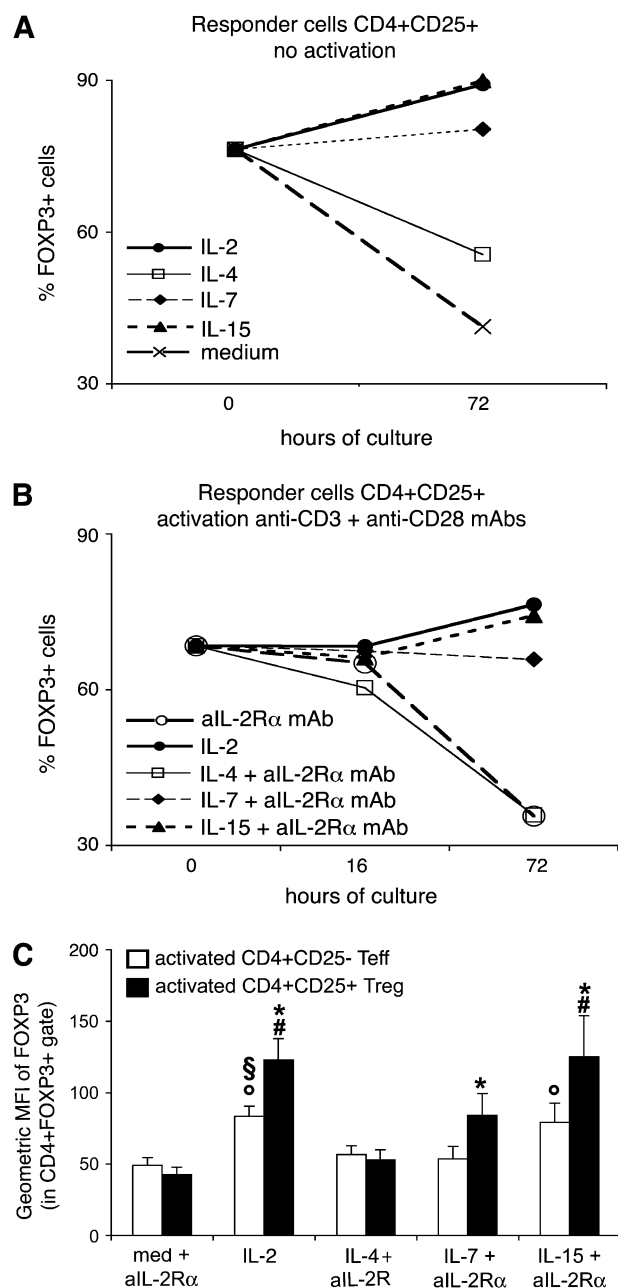


Fig. 4. Maintenance of FOXP3 expression in CD4⁺CD25⁺ T cells is STAT5-signaling cytokine dependent. (A) CD4⁺CD25⁺ T cells were cultured without activation for 72 h either alone or in the presence of alternatively IL-2, IL-4, IL-7 or IL-15. (B) CD4⁺CD25⁺ T cells were activated in the presence of plate-bound anti-CD3, soluble anti-CD28 mAbs in combination with IL-2, anti-IL-2Rα mAb alone or plus alternatively IL-4, IL-7 or IL-15. At the indicated time points, cells were stained to determine FOXP3 expression. Percentages of FOXP3⁺ T cells in the CD4⁺ gate are plotted in the graph (one representative donor). (C) CD4⁺CD25⁺ Treg cells (black bars) and CD4⁺CD25⁻ Teff cells (white bars) were activated in the presence of IL-2, anti-IL-2Rα mAb alone or in combination with IL-4, IL-7 or IL-15; after 72 h, T cells were stained to determine FOXP3 expression. Mean geometric MFI $\pm$ SE in CD4⁺FOXP3⁺ gate is reported in the graph. For each condition at least five experiments were performed. Statistical analysis was performed with the unpaired Student's *t*-test. **P* < 0.05 versus Treg med + aIL-2Rα; #*P* < 0.05 versus Treg IL-4 + aIL-2Rα; °*P* < 0.05 versus Teff med + aIL-2Rα and §*P* < 0.05 versus Teff IL-4 + aIL-2Rα.

We have recently shown that stable ectopic expression of FOXP3 in human CD4⁺CD25⁻ cells is sufficient to induce regulatory function (32). To test whether the levels of FOXP3 induced upon constitutive STAT5a signaling also conferred a regulatory phenotype, we monitored whether transduced T cells acquired suppressive capacity *in vitro*. Despite elevated levels of FOXP3, STAT5a-CA-transduced cells were hyperproliferative compared with control-transduced cells (Fig. 3B) and did not have the capacity to suppress responder T cells (Fig. 3C). These results demonstrate that, while FOXP3 expression is induced by STAT5, other effects of constitutive STAT5 signaling override the capacity of FOXP3 to induce a hyporesponsive and suppressive function in Teff cells.

High FOXP3 expression in CD4⁺CD25⁺ Treg cells is maintained in the presence of STAT5-signaling cytokines

It is generally thought that IL-2 is essential for the survival and function of CD4⁺CD25⁺ Treg cells in the periphery (2, 3, 44, 45). We therefore investigated whether IL-2 was important for maintaining high FOXP3 expression in Treg cells. The effects of IL-7 and IL-15 were tested in parallel. After 72 h of culture, the viability of Treg cells was significantly better in the presence of IL-2, IL-7 or IL-15 compared with cells cultured in medium alone or with IL-4 (Supplementary Figure 1B, available at *International Immunology* Online). However, upon analysis of gated cells, the viability was >95% in all culture condition (Supplementary Figure 1A and C, available at *International Immunology* Online). In live cell gate (Supplementary Figure 1A, available at *International Immunology* Online), we found that FOXP3 expression was significantly down-regulated in Treg cells cultured in medium alone (*n* = 7) or with IL-4 (*n* = 14) (*P* < 0.05 versus T0), whereas it was maintained in the presence of IL-7 (*n* = 11, *P* = NS versus T0) and increased in the presence of IL-2 (*n* = 14) or IL-15 (*n* = 11) (*P* < 0.03 versus T0) (Fig. 4A and Table 2). Addition of TGF-β to the culture medium was not sufficient to maintain high FOXP3 expression (data not shown). Similar to Teff cells, TCR-mediated activation of Treg cells in the presence of exogenous IL-2 induced an increase in FOXP3 expression (Fig. 4B and Table 3) and blockade of the IL-2R by addition of an anti-IL-2Rα mAb resulted in decreased expression. In these culture conditions, addition of IL-4 did not rescue the decrease in FOXP3 expression in the absence of IL-2 signaling. In contrast, IL-15, and to a lesser extent IL-7, maintained FOXP3 expression even when the IL-2R was blocked (Fig. 4B and Table 3).

Analysis of the intensity of FOXP3 expression at the single cell level (as measured by MFI) confirmed that STAT5-activating cytokines maintain FOXP3 expression in Treg cells. The MFI of FOXP3 was significantly higher in Treg cells activated in the presence of IL-2 and IL-15, and to a lesser extent with IL-7, compared with cells activated in medium or with IL-4 and the IL-2R-blocking mAb (Fig. 4C). In agreement with our previous data (27), the levels of FOXP3 expression in Teff cell expression never reached those detected in activated Treg cells (Fig. 4C). These observations indicate that STAT5-activating cytokines positively influence FOXP3 expression in both CD4⁺CD25⁺ Treg and activated CD4⁺CD25⁻ Teff cells.

Table 2. Percentage of FOXP3⁺ cells in cultured CD4⁺CD25⁺ T cells

Culture conditions	FOXP3 expression ^a (mean % ± SE)	n	P ^b versus IL-4	P ^c versus T0
Medium	25.2 ± 4.7	7	<0.05	<0.05
IL-2	69.1 ± 5.7	14	<0.05	<0.05
IL-4	47.3 ± 4.7	14	<0.05	<0.05
IL-7	61.0 ± 6.3	11	NS	NS
IL-15	65.4 ± 6.2	11	<0.05	<0.05

NS, not significant.

^aValues refer to 72 h cultures.^bStatistical analysis was performed with the unpaired Student's *t*-test.^cStatistical analysis was performed with the paired Student's *t*-test.**Table 3.** Percentage of FOXP3⁺ cells in activated CD4⁺CD25⁺ T cells

Culture conditions	FOXP3 expression ^b (mean % ± SE)	n	P ^c versus IL-4	P ^d versus T0
IL-2	51.4 ± 5.4	7	<0.005	NS
IL-4 + aIL-2Rα	24.4 ± 4.4	7		<0.005
aIL-2Rα	22.9 ± 5.0	5	NS	<0.05
IL-7 + aIL-2Rα	37.4 ± 6.1	5	NS	NS
IL-15 + aIL-2Rα	48.2 ± 7.4	5	<0.05	NS

aIL-2Rα, anti-IL-2Rα mAb; NS, not significant.

^aActivation: anti-CD3 + anti-CD28 mAbs.^bValues refer to 72 h cultures.^cStatistical analysis was performed with the unpaired Student's *t*-test.^dStatistical analysis was performed with the paired Student's *t*-test.

Activation of the PI3K pathway is not required for sustained FOXP3 expression in CD4⁺CD25⁺ T cells

To further examine the molecular pathways required to maintain expression of FOXP3 in Treg cells, we compared the effects of inhibiting the Jak/STAT5 pathway (29), or the PI3K pathway, by addition of rapamycin (30). As above (Fig. 4A), constitutive expression of FOXP3 in CD4⁺CD25⁺ Treg cells was maintained in culture with IL-2, IL-7 or IL-15, but not IL-4 (Fig. 5). As expected, addition of the Jak inhibitor to Treg cell cultures prevented cytokine-induced maintenance of FOXP3 expression (Fig. 5) ($P < 0.05$ versus cytokine alone). In contrast, culture in the presence of rapamycin did not prevent the capacity of cytokines to maintain FOXP3 expression in Treg cells (Fig. 5). These results further support the hypothesis that FOXP3 expression is regulated by STAT5 and not by the PI3K pathway.

IL-2 maintains the in vitro suppressive capacity of CD4⁺CD25⁺ Treg cells

In order to investigate whether STAT5-signaling cytokines enhanced the suppressive activity of CD4⁺CD25⁺ Treg cells or induced regulatory function in CD4⁺CD25⁺ Teff cells, we tested the suppressive capacity of both Treg and Teff cells after 72 h of culture in the presence of IL-2, IL-4, IL-7 or IL-15.

After 3 days of culture in different conditions, CD4⁺CD25⁺ Treg cells were tested in a suppression assay using autologous CD4⁺CD25⁺ T cells as responder cells. Treg cells cultured in the presence of exogenous IL-2 suppressed the

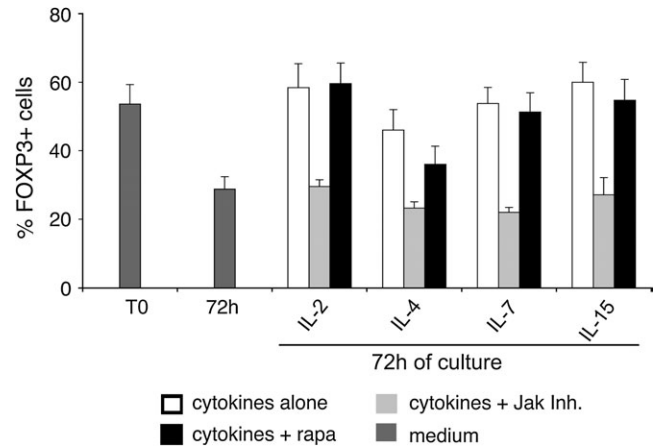


Fig. 5. FOXP3 up-regulation in CD4⁺CD25⁺ Treg cells is STAT5 dependent. CD4⁺CD25⁺ T cells were cultured without activation with IL-2, IL-4, IL-7 or IL-15 in the presence or absence of a Jak inhibitor (Jak Inh) (mean reduction %FOXP3⁺ ± SE in the presence of IL-2 plus Jak Inh was 23.21 ± 3.4, $n = 5$; in the presence of IL-7 plus Jak Inh was 33.3 ± 5.9, $n = 4$; in the presence of IL-15 plus Jak Inh was 28.1 ± 3.3, $n = 4$ and in the presence of IL-4 plus Jak Inh was 29.4 ± 4.8, $n = 5$) or rapamycin (mean Δ%FOXP3⁺ ± SE in the presence of IL-2 plus rapamycin was +6.9 ± 1.9, $n = 5$; in the presence of IL-7 plus rapamycin was −1.4 ± 2.8, $n = 4$; in the presence of IL-15 plus rapamycin was +1.9 ± 2.8, $n = 4$ and in the presence of IL-4 plus rapamycin was −16.7 ± 3.3, $n = 5$). FOXP3 expression was determined by FACS analysis at T0 and after 72 h of culture. Mean percentage of FOXP3⁺ T cells in the CD4⁺ gate (±SE) is plotted in the graph.

proliferation of responder cells with an efficiency comparable to freshly isolated CD4⁺CD25⁺ Treg cells (Fig. 6A and B). Conversely, Treg cells cultured in medium alone decreased their suppressive capacity (Fig. 6B). Similarly, Treg cells cultured with IL-4 displayed reduced suppressive capacity in six out of seven experiments, when compared with Treg cells cultured in IL-2 ($P = 0.008$) (Fig. 6A and B). Comparable results were obtained using FACS-sorted CD4⁺CD25⁺ T cells (data not shown). Similar to IL-2-treated Treg cells, CD4⁺CD25⁺ T cells cultured in the presence of IL-15 maintained high suppressive capacity, whereas IL-7 was less efficient (Fig. 6A and B). Overall, these results demonstrate a correlation between the levels of FOXP3 expression and suppressive capacity.

CD4⁺CD25⁺ Teff cells activated in the presence of IL-2, IL-7 or IL-15 up-regulated FOXP3 expression after 72 h, but did not display *in vitro* suppressive function (Fig. 6C), in line with recent reports (24, 27). The lack of regulatory function by activated FOXP3⁺ Teff cells could be attributed to lower amounts of FOXP3 expression per cell compared with Treg cells (Fig. 4C). Alternatively, other signaling cascades activated in Teff cells may override the capacity of FOXP3 to induce a regulatory phenotype. This latter possibility is in agreement with data shown in Fig. 3C, in which STAT5a-CA-transduced cells show a hyperproliferative capacity despite a significant increase in FOXP3 expression.

Discussion

In the present work, we demonstrate that expression of FOXP3 in TCR-activated human CD4⁺CD25⁺ Teff cells requires cytokine-mediated activation of STAT5. A direct role for STAT5 in regulating FOXP3 expression was demonstrated

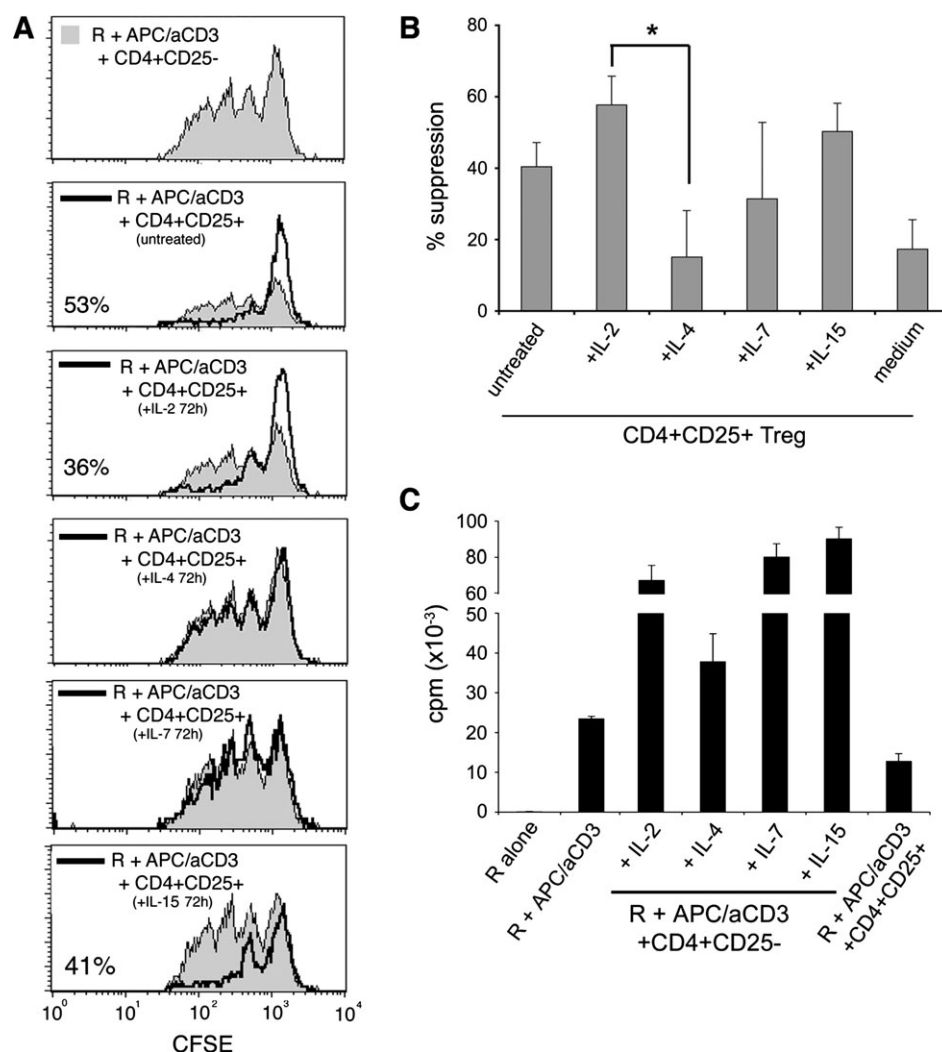


Fig. 6. Maintenance of suppressive activity in CD4⁺CD25⁺ T cells is IL-2 dependent. (A and B) After 72 h of culture with IL-2 ($n = 7$), IL-4 ($n = 7$), IL-7 ($n = 3$), IL-15 ($n = 3$) or medium ($n = 4$), CD4⁺CD25⁺ T cells were used as suppressor cells in a CFSE dilution assay, using autologous CD4⁺CD25⁺ T cells as responder cells (R). As positive control for suppression, autologous CD4⁺CD25⁺ T cells, indicated as untreated ($n = 7$), were used. (A) Histograms of CFSE dilution of one representative donor; numbers in the plots indicate percent inhibition. (B) Average percent inhibition of proliferation $\pm$ SE is plotted in the graph. * $P < 0.05$ versus IL-2-treated CD4⁺CD25⁺ Treg cells. Statistical analysis was performed using the paired Student's *t*-test. Statistical analysis was not performed for experimental groups including less than five independent repeats. (C) After 72 h of activation with anti-CD3 and anti-CD28 mAbs plus alternatively IL-2, IL-4, IL-7 or IL-15 CD4⁺CD25⁺ T cells were used as suppressor cells in a suppression assay, using autologous CD4⁺CD25⁺ T cells as responder cells. As positive control for suppression, autologous CD4⁺CD25⁺ Treg cells were used. Numbers in the plots indicate percent inhibition (representative of five donors for IL-2- and IL-4-treated Teff cells and three donors for IL-7- and IL-15-treated Teff cells).

by gene transfer of a constitutively active form of STAT5-CA. In no case, however, was activation-induced FOXP3 expression in Teff cells sufficient to confer suppressive function to these cells. Similar to Teff cells, STAT5-signaling cytokines also positively regulate FOXP3 expression in human CD4⁺CD25⁺ Treg cells. Withdrawal of STAT5-activating cytokines caused a decrease in FOXP3 expression and a subsequent reduction in the suppressive capacity of cultured Treg cells. Overall, these data indicate that FOXP3 expression is regulated by similar mechanisms in both peripheral Treg and Teff cells, but that other signaling pathways play an essential role in determining regulatory function in Treg cells.

In agreement with recent reports, we confirm that FOXP3 can be induced upon activation of human Teff cells (24, 25,

27, 34). We also provide new evidence that blockade of the IL-2R α in activated human Teff cells prevents induction of FOXP3 and that FOXP3 expression in resting CD4⁺CD25⁺ T cells is not induced in response to cytokines in the absence of TCR activation. Thus, signaling pathways activated by the TCR and STAT5 are required for activation-induced FOXP3 expression. In addition, although STAT5-activating cytokines are growth factors that stimulate *in vitro* proliferation of Teff cells, activation-induced FOXP3 expression occurs independently of cell division.

Importantly, we show that the capacity to induce FOXP3 expression in activated Teff cells is not a unique property of IL-2, but it is also shared by IL-7 and IL-15, other STAT5-activating cytokines. This novel observation for human T cells

differs from what has been described for murine CD4⁺CD25⁻ T cells (46, 47), in which IL-7 and IL-15 cannot substitute for IL-2 in induction of TGF- β -stimulated FOXP3 expression. Thus, mouse and human Teff cells may have a differential requirement for STAT5 activity for induction of FOXP3 expression. In murine Teff cells, FOXP3 induction only occurs in the presence of exogenous TGF- β (46, 48), but it has been demonstrated that STAT5 plays a non-redundant role in TGF- β -mediated FOXP3 induction even in murine T cells (48). STAT5 dependency of FOXP3 expression in human Treg cells has previously been described (20–22). Here, we provide evidence of a direct role for STAT5 in the regulation of FOXP3 expression in non-Treg cell compartment.

IL-2, IL-15 and IL-4 are growth factors which sustain *in vitro* proliferation of Teff cells (1, 49, 50), but only IL-2 and IL-15, which signal through STAT5, can induce FOXP3 expression in activated Teff cells. Therefore, although FOXP3 can be detected in proliferating cells (27), progression through the cycle is not the driving force for FOXP3 expression. Indeed, in the presence of the anti-proliferative drug, rapamycin, expression of FOXP3 can still be induced.

A controversial issue is whether Teff cells acquire regulatory function upon activation-induced expression of FOXP3. We show that neither cytokine- nor STAT5-CA-induced FOXP3 expression confers regulatory activity to CD4⁺CD25⁻ T cells. This is in accordance with recently published data, showing that activation-induced FOXP3 neither suppressed cytokine production in Teff cells (26, 27) nor induced suppressive activity in CD4⁺CD25⁻ T cells (24). Similarly, it has been reported that TGF- β -induced FOXP3 expression does not give rise to a suppressive population of T cells (41). FOXP3 expression in Teff cells is transient and never reaches levels observed in Treg cells, supporting the notion that high and stable levels of FOXP3 are required for acquisition and maintenance of regulatory capacity by T cells (51, 52). Nonetheless, it cannot be excluded that prolonged culture (34) with selective enrichment of FOXP3⁺ T cells could eventually confer a suppressive function to non-Treg cells, possibly through stabilization of FOXP3 expression in the resulting population.

The fact that IL-4 or TGF- β alone do not up-regulate FOXP3 expression in Teff cells suggests that activation of STAT6 or SMAD is not sufficient to induce FOXP3 expression. Although it has been reported that TGF- β can induce FOXP3 expression in activated human Teff cells (40, 41, 53), other reports in human and mouse T cells show that TGF- β -induced FOXP3 expression requires exogenous IL-2 (46–48, 54). Our data demonstrating that TGF- β is not sufficient to induce FOXP3 expression in human Teff cells, contrast with a recently published report by Tran *et al.* (41). Tran *et al.* demonstrated that TGF- β is required for induction of FOXP3 expression, particularly in naive CD4⁺CD25⁻CD45RA⁺ T cells, and less so in memory CD4⁺CD25⁻CD45RA⁻ T cells. Since our experiments were performed with CD4⁺CD25⁻ T cells, which primarily include memory T cells, the discrepancy is likely related to differences in the proportions of memory versus naive T cells in the cultures. We can also speculate that TGF- β may not directly regulate FOXP3 expression, but that it might be important

for the stabilization of FOXP3 expression or for the survival of FOXP3 expressing cells in long-term culture.

As previously described (20, 21), we found that IL-2 is necessary to sustain FOXP3 expression in human CD4⁺CD25⁺ Treg cells. Through maintenance of high FOXP3 expression in Treg cells, IL-2 preserves their suppressive capacity, which is otherwise lost in culture. The capacity of IL-2 to maintain FOXP3 expression is shared by other STAT5-signaling cytokines, in particular IL-15, suggesting that IL-2 can be replaced by IL-15, at least *in vitro*. Indeed, it has recently been demonstrated that the proliferation of Treg cells can be sustained *in vitro* by IL-15 and dermal fibroblasts, mirroring the local expansion of natural Treg cells within inflamed tissues (55). Thus, optimal conditions for *in vitro* expansion of human Treg cells should include cytokines which activate STAT5.

Among the STAT5-signaling cytokines tested, IL-2 and IL-15 share the same potency in inducing/maintaining FOXP3 expression, whereas IL-7 was less efficient. This is in line with the reported low expression of IL-7R (CD127) in peripheral CD4⁺CD25⁺ Treg cells (56) and with down-regulation of IL-7R in activated CD4⁺CD25⁻ Teff cells (27). Interestingly, IL-2 and IL-15 also share the usage of the β -chain (12), suggesting that the β -chain may play an additional role in the induction/maintenance of FOXP3 expression in Teff/Treg cells. This is in accordance with recent reports showing that both in IL-2R β -deficient mice (18, 57) and in IL-2 and IL-15 double knockout mice (58), Treg cell differentiation, maintenance and function are impaired.

At present, the differential role for FOXP3 in Treg and Teff cells and how the STAT5-signaling cytokines differentially contribute to the generation/function of Treg and Teff cells remains to be addressed.

STAT5 signaling is important for FOXP3 expression and Treg cell function also *in vivo*, as T cells from patients with a mutation in the *STAT5* gene lack FOXP3 expression and have impaired suppressive activity (22). In contrast, in patients with *STAT3* mutations, FOXP3 is not reported to be differentially expressed as compared with control donors, suggesting that STAT3 does not affect FOXP3 expression (59). Further knowledge of the molecular mechanisms controlling FOXP3 expression and Treg cell expansion and function will be instrumental in the development of strategies to use these cells for therapeutic purposes.

Supplementary data

Supplementary Figure 1 available at *International Immunology* Online (www.intimm.oxfordjournals.org).

Funding

Italian Telethon Foundation (Tele6A3 and GGP04285) to R.B.; Canadian Institutes for Health Research (MOP127506) to M.K.L.; Michael Smith Foundation for Health Research (CI-SCH-18(02-1)) to M.K.L.; Michael Smith Foundation for Health Research Senior Graduate Studentship Award (ST-SGS-396(06-1)) and Canadian Institutes for Health Research Canada Graduate Scholarship Doctoral Award (CGD-80386) to S.E.A.

Acknowledgements

We thank H. Spitz for critical discussion. M.K.L. holds a Canada Research Chair in Transplantation.

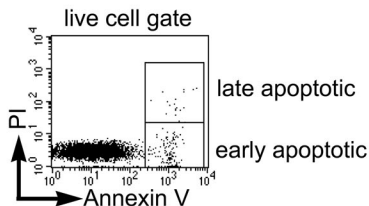
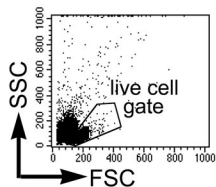
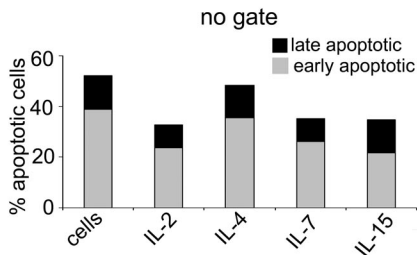
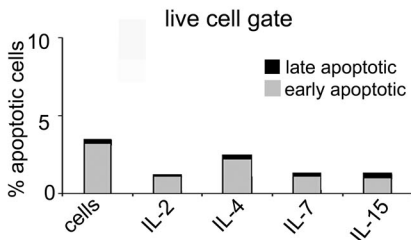
Abbreviations

APC	antigen-presenting cell
CFSE	carboxyfluorescein diacetate succinimidylester
γ C	γ -chain
FOXP3	forkhead box P3
MFI	mean fluorescence intensity
P13K	phosphatidylinositol-3-kinase
SE	standard error
STAT5	signal transducer and activator of transcription 5
STAT5a-CA	constitutively active signal transducer and activator of transcription 5a
Teff	effector T
TGF- β	transforming growth factor- β
Treg	regulatory T

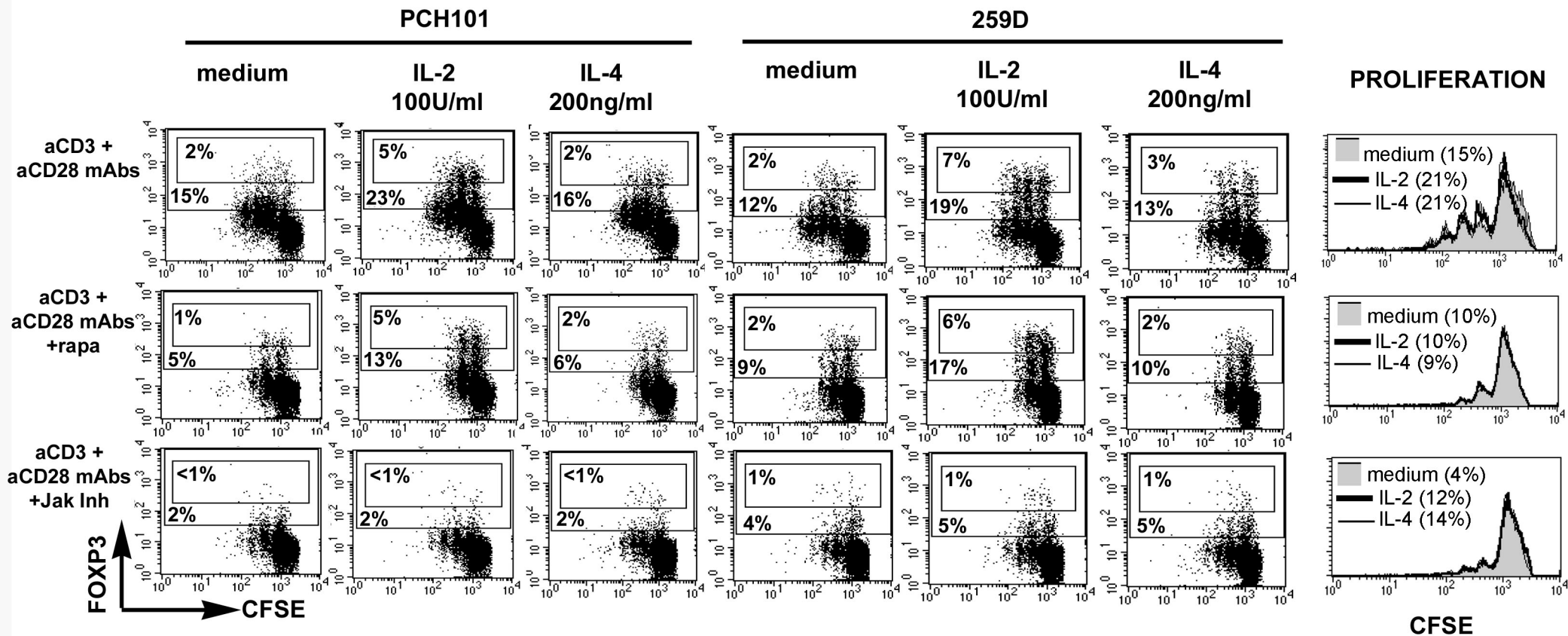
References

- Smith, K. A. 1988. Interleukin-2: inception, impact, and implications. *Science* 240:1169.
- Setoguchi, R., Hori, S., Takahashi, T. and Sakaguchi, S. 2005. Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J. Exp. Med.* 201:723.
- Thornton, A. M., Donovan, E. E., Piccirillo, C. A. and Shevach, E. M. 2004. Cutting edge: IL-2 is critically required for the *in vitro* activation of CD4+CD25+ T cell suppressor function. *J. Immunol.* 172:6519.
- Malek, T. R. and Bayer, A. L. 2004. Tolerance, not immunity, crucially depends on IL-2. *Nat. Rev. Immunol.* 4:665.
- Sadlack, B., Merz, H., Schorle, H., Schimpl, A., Feller, A. C. and Horak, I. 1993. Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. *Cell* 75:253.
- Poussier, P., Ning, T., Chen, J., Banerjee, D. and Julius, M. 2000. Intestinal inflammation observed in IL-2R/IL-2 mutant mice is associated with impaired intestinal T lymphopoiesis. *Gastroenterology* 118:880.
- Willerford, D. M., Chen, J., Ferry, J. A., Davidson, L., Ma, A. and Alt, F. W. 1995. Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment. *Immunity* 3:521.
- D'Cruz, L. M. and Klein, L. 2005. Development and function of agonist-induced CD25+Foxp3+ regulatory T cells in the absence of interleukin 2 signaling. *Nat. Immunol.* 6:1152.
- Fontenot, J. D., Rasmussen, J. P., Gavin, M. A. and Rudensky, A. Y. 2005. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat. Immunol.* 6:1142.
- Nelson, B. H. and Willerford, D. M. 1998. Biology of the interleukin-2 receptor. *Adv. Immunol.* 70:1.
- Kim, H. P., Imbert, J. and Leonard, W. J. 2006. Both integrated and differential regulation of components of the IL-2/IL-2 receptor system. *Cytokine Growth Factor Rev.* 17:349.
- Giri, J. G., Ahdieh, M., Eisenman, J. *et al.* 1994. Utilization of the beta and gamma chains of the IL-2 receptor by the novel cytokine IL-15. *EMBO J.* 13:2822.
- He, Y. W. and Malek, T. R. 1998. The structure and function of gamma c-dependent cytokines and receptors: regulation of T lymphocyte development and homeostasis. *Crit. Rev. Immunol.* 18:503.
- Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. and Toda, M. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 155:1151.
- Fontenot, J. D., Rasmussen, J. P., Williams, L. M., Dooley, J. L., Farr, A. G. and Rudensky, A. Y. 2005. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 22:329.
- Shevach, E. M. 2002. CD4+ CD25+ suppressor T cells: more questions than answers. *Nat. Rev. Immunol.* 2:389.
- Wan, Y. Y. and Flavell, R. A. 2006. The roles for cytokines in the generation and maintenance of regulatory T cells. *Immunol. Rev.* 212:114.
- Malek, T. R., Yu, A., Vincek, V., Scibelli, P. and Kong, L. 2002. CD4 regulatory T cells prevent lethal autoimmunity in IL-2Rbeta-deficient mice. Implications for the nonredundant function of IL-2. *Immunity* 17:167.
- Hori, S., Nomura, T. and Sakaguchi, S. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299:1057.
- Zorn, E., Nelson, E. A., Mohseni, M. *et al.* 2006. IL-2 regulates FOXP3 expression in human CD4+CD25+ regulatory T cells through a STAT-dependent mechanism and induces the expansion of these cells *in vivo*. *Blood* 108:1571.
- Murawski, M. R., Litherland, S. A., Clare-Salzler, M. J. and Davoodi-Semiromi, A. 2006. Upregulation of Foxp3 expression in mouse and human Treg is IL-2/STAT5 dependent: implications for the NOD STAT5B mutation in diabetes pathogenesis. *Ann. N Y Acad. Sci.* 1079:198.
- Cohen, A. C., Nadeau, K. C., Tu, W. *et al.* 2006. Cutting edge: decreased accumulation and regulatory function of CD4+ CD25(high) T cells in human STAT5b deficiency. *J. Immunol.* 177:2770.
- Allan, S. E., Passerini, L., Bacchetta, R. *et al.* 2005. The role of 2 FOXP3 isoforms in the generation of human CD4+ Tregs. *J. Clin. Invest.* 115:3276.
- Wang, J., Ioan-Facsinay, A., van der Voort, E. I., Huizinga, T. W. and Toes, R. E. 2007. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *Eur. J. Immunol.* 37:129.
- Pillai, V., Ortega, S. B., Wang, C. K. and Karandikar, N. J. 2006. Transient regulatory T-cells: a state attained by all activated human T-cells. *Clin Immunol.* 123:18.
- Gavin, M. A., Torgerson, T. R., Houston, E. *et al.* 2006. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proc. Natl Acad. Sci. USA* 103:6659.
- Allan, S. E., Crome, S. Q., Crellin, N. K. *et al.* 2007. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. *Int. Immunol.* 19:345.
- Lyons, A. B. and Parish, C. R. 1994. Determination of lymphocyte division by flow cytometry. *J. Immunol. Methods* 171:131.
- Thompson, J. E., Cubbon, R. M., Cummings, R. T. *et al.* 2002. Photochemical preparation of a pyridone containing tetracycline: a Jak protein kinase inhibitor. *Bioorg. Med. Chem. Lett.* 12:1219.
- Abraham, R. T. and Wiederrecht, G. J. 1996. Immunopharmacology of rapamycin. *Annu. Rev. Immunol.* 14:483.
- Onishi, M., Nosaka, T., Misawa, K. *et al.* 1998. Identification and characterization of a constitutively active STAT5 mutant that promotes cell proliferation. *Mol. Cell. Biol.* 18:3871.
- Allan, S. E., Alstad, A. N., Merindol, N. *et al.* 2007. Generation of potent and stable human CD4(+) T regulatory cells by activation-independent expression of FOXP3. *Mol. Ther.* 16:194.
- Amendola, M., Venneri, M. A., Biffi, A., Vigna, E. and Naldini, L. 2005. Coordinate dual-gene transgenesis by lentiviral vectors carrying synthetic bidirectional promoters. *Nat. Biotechnol.* 23:108.
- Walker, M. R., Kaspruwicz, D. J., Gersuk, V. H. *et al.* 2003. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells. *J. Clin. Invest.* 112:1437.
- Kovanen, P. E. and Leonard, W. J. 2004. Cytokines and immunodeficiency diseases: critical roles of the gamma(c)-dependent cytokines interleukins 2, 4, 7, 9, 15, and 21, and their signaling pathways. *Immunol. Rev.* 202:67.
- Paukku, K. and Silvennoinen, O. 2004. STATs as critical mediators of signal transduction and transcription: lessons learned from STAT5. *Cytokine Growth Factor Rev.* 15:435.
- Chatila, T. A. 2004. Interleukin-4 receptor signaling pathways in asthma pathogenesis. *Trends Mol. Med.* 10:493.
- Fantini, M. C., Becker, C., Tubbe, I. *et al.* 2006. Transforming growth factor beta induced FoxP3+ regulatory T cells suppress Th1 mediated experimental colitis. *Gut* 55:671.

- 39 Chen, W., Jin, W., Hardegen, N. *et al.* 2003. Conversion of peripheral CD4+CD25⁻ naive T cells to CD4+CD25⁺ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J. Exp. Med.* 198:1875.
- 40 Fantini, M. C., Becker, C., Monteleone, G., Pallone, F., Galle, P. R. and Neurath, M. F. 2004. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25⁻ T cells through Foxp3 induction and down-regulation of Smad7. *J. Immunol.* 172:5149.
- 41 Tran, D. Q., Ramsey, H. and Shevach, E. M. 2007. Induction of FOXP3 expression in naive human CD4+FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype. *Blood* 110:2983.
- 42 John, S., Robbins, C. M. and Leonard, W. J. 1996. An IL-2 response element in the human IL-2 receptor alpha chain promoter is a composite element that binds Stat5, Elf-1, HMG-I(Y) and a GATA family protein. *EMBO J.* 15:5627.
- 43 Lecine, P., Algarte, M., Rameil, P. *et al.* 1996. Elf-1 and Stat5 bind to a critical element in a new enhancer of the human interleukin-2 receptor alpha gene. *Mol. Cell. Biol.* 16:6829.
- 44 Furtado, G. C., Curotto de Lafaille, M. A., Kutchukhidze, N. and Lafaille, J. J. 2002. Interleukin 2 signaling is required for CD4(+) regulatory T cell function. *J. Exp. Med.* 196:851.
- 45 de la Rosa, M., Rutz, S., Dorninger, H. and Scheffold, A. 2004. Interleukin-2 is essential for CD4+CD25+ regulatory T cell function. *Eur. J. Immunol.* 34:2480.
- 46 Zheng, S. G., Wang, J., Wang, P., Gray, J. D. and Horwitz, D. A. 2007. IL-2 is essential for TGF-beta to convert naive CD4+CD25⁻ cells to CD25+Foxp3+ regulatory T cells and for expansion of these cells. *J. Immunol.* 178:2018.
- 47 Davidson, T. S., DiPaolo, R. J., Andersson, J. and Shevach, E. M. 2007. Cutting edge: IL-2 is essential for TGF-beta-mediated induction of Foxp3+ T regulatory cells. *J. Immunol.* 178:4022.
- 48 Yao, Z., Kanno, Y., Kerenyi, M. *et al.* 2007. Nonredundant roles for Stat5a/b in directly regulating Foxp3. *Blood*.
- 49 Hu-Li, J., Shevach, E. M., Mizuguchi, J., Ohara, J., Mosmann, T. and Paul, W. E. 1987. B cell stimulatory factor 1 (interleukin 4) is a potent costimulant for normal resting T lymphocytes. *J. Exp. Med.* 165:157.
- 50 Bacchetta, R., Sartirana, C., Levings, M. K., Bordinon, C., Narula, S. and Roncarolo, M. G. 2002. Growth and expansion of human T regulatory type 1 cells are independent from TCR activation but require exogenous cytokines. *Eur. J. Immunol.* 32:2237.
- 51 Wan, Y. Y. and Flavell, R. A. 2007. Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. *Nature* 445:766.
- 52 Williams, L. M. and Rudensky, A. Y. 2007. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nat. Immunol.* 8:277.
- 53 Zheng, S. G., Wang, J. H., Gray, J. D., Soucier, H. and Horwitz, D. A. 2004. Natural and induced CD4+CD25+ cells educate CD4+CD25⁻ cells to develop suppressive activity: the role of IL-2, TGF-beta, and IL-10. *J. Immunol.* 172:5213.
- 54 Horwitz, D. A., Zheng, S. G. and Gray, J. D. 2003. The role of the combination of IL-2 and TGF-beta or IL-10 in the generation and function of CD4+ CD25+ and CD8+ regulatory T cell subsets. *J. Leukoc. Biol.* 74:471.
- 55 Clark, R. A. and Kupper, T. S. 2007. IL-15 and dermal fibroblasts induce proliferation of natural regulatory T cells isolated from human skin. *Blood* 109:194.
- 56 Liu, W., Putnam, A. L., Xu-Yu, Z. *et al.* 2006. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J. Exp. Med.* 203:1701.
- 57 Soper, D. M., Kaspirowicz, D. J. and Ziegler, S. F. 2007. IL-2Rbeta links IL-2R signaling with Foxp3 expression. *Eur. J. Immunol.* 37:1817.
- 58 Burchill, M. A., Yang, J., Vogtenhuber, C., Blazar, B. R. and Farrar, M. A. 2007. IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3+ regulatory T cells. *J. Immunol.* 178:280.
- 59 Holland, S. M., DeLeo, F. R., Elloumi, H. Z. *et al.* 2007. STAT3 mutations in the hyper-IgE syndrome. *N. Engl. J. Med.* 357:1608.

A**B****C**

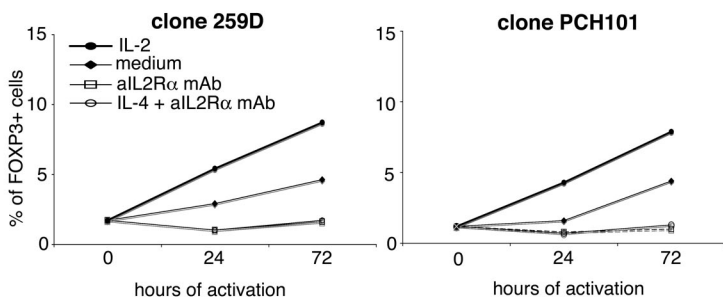
Supplementary Figure 1. Treg cell viability. Treg cells were cultured either alone or in the presence of cytokines (IL-2, IL-4, IL-7 or IL-15). After 72h percentage of early and late apoptotic cells was determined by Annexin V/PI staining. (A) Percentage of apoptotic cells was measured in the live cell gate (upper panel); Annexin V⁺ cells were identified as apoptotic cells, whereas PI staining was used to distinguish early and late apoptotic populations (lower panel). One representative staining is shown. (B and C) Percentage of early and late apoptotic cells either without gating (B) or in the live cell gate (C) in the different culture conditions is plotted in the graph (representative of n=3 donors).



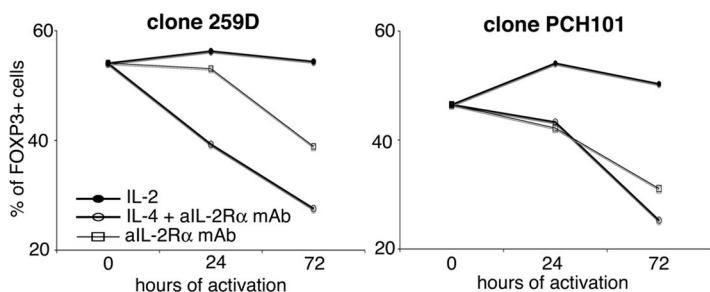
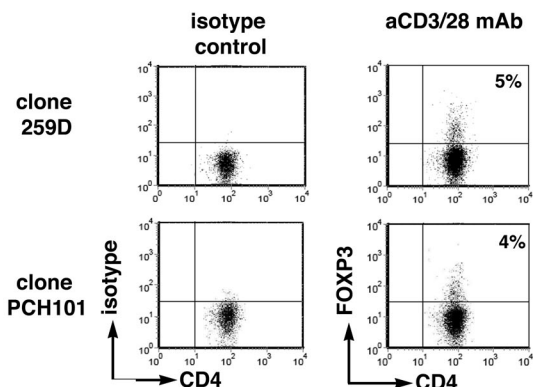
SFigure 2. FOXP3 expression and proliferation: comparative study of anti-human FOXP3 mAbs (PCH101 clone and 259D clone). CFSE labeled CD4⁺CD25⁻ Teff cells were activated in the presence of anti-CD3 plus anti-CD28 mAbs. After 24h of activation, alternatively IL-2 or IL-4, alone or in combination with either rapamycin (rapa) or a Jak Inhibitor (Jak Inh) were added to the culture medium. After 72h the cells were stained to determine FOXP3 expression either with clone PCH101 (left panels) or with clone 259D (right panels). Region of positive staining was set based on the relative isotype control. Percentages of FOXP3⁺ (large frame) or FOXP3^{bright} (small frame) cells are reported in the plots. Histograms show CFSE dilutions of untreated (grey peaks) or IL-2- (bold line)/IL-4- (thin line) treated samples either alone (upper panel) or in the presence of rapamycin (middle panel)/Jak Inhibitor (lower panel). Numbers in the histogram plots indicate percent of proliferating precursors (representative of three donors).

A

Responder cells CD4⁺CD25⁻
activation: anti-CD3 + anti-CD28 mAb

**B**

Responder cells CD4⁺CD25⁺
activation: anti-CD3 + anti-CD28 mAb

**C**

SFigure 3. (A) CD4⁺CD25⁻ T cells were activated in the presence of plate-bound anti-CD3, soluble anti-CD28 mAbs either alone or in combination with IL-2, or anti-IL-2R α mAb with or without IL4 (one representative donor out of 3). (B) CD4⁺CD25⁺ T cells were activated in the presence of plate-bound anti-CD3, soluble anti-CD28 mAbs in combination with either IL-2, anti-IL-2R α mAb alone or plus IL-4 (one representative donor out of 2). After 24-72h of incubation, T cells were stained to determine FOXP3 expression with anti-FOXP3 mAb PCH101 clone or 259D clone. Percentages of FOXP3⁺ T cells in the CD4⁺ gate measured at each time point are plotted in the graphs. (C) CD4⁺CD25⁻ T cells were activated in the presence of plate-bound anti-CD3 and soluble anti-CD28 mAbs. Representative dot plots of CD4/FOXP3 staining obtained on the same donor with either anti-FOXP3 mAb 259D clone or PCH101 clone are shown.

STable 1: Percentage of FOXP3⁺ cells in activated* CD4⁺CD25⁻ T cells in the presence or absence of STAT/PI3K pathway inhibitors, after 4 days of culture.

Donor	culture conditions	%FOXP3 ⁺ (PCH 101)			%FOXP3 ⁺ (259D)		
		medium	IL-2	IL-4	medium	IL-2	IL-4
89	aCD3+aCD28 mAbs	15	23	16	12	19	13
89	aCD3+aCD28 mAbs +rapa	5	13	6	9	17	10
89	aCD3+aCD28 mAbs +Jak Inh	2	2	2	4	5	5
63	aCD3+aCD28 mAbs	3	11	5	4	13	3
63	aCD3+aCD28 mAbs +rapa	2	11	3	3	11	2
63	aCD3+aCD28 mAbs +Jak Inh	1	1	<1	1	<1	1
18	aCD3+aCD28 mAbs	6	16	4	6	12	6
18	aCD3+aCD28 mAbs +rapa	3	6	3	4	9	4
18	aCD3+aCD28 mAbs +Jak Inh	1	1	1	<1	1	1

*activation: anti-CD3+ anti-CD28 mAbs;