FOXP3 Induced by CD28/B7 Interaction Regulates CD25 and Anergic Phenotype in Human CD4+CD25− T Lymphocytes

Cristiano Scottà, Marzia Soligo, Cristina Camperio and Enza Piccolella

*J Immunol* 2008; 181:1025-1033;
doi: 10.4049/jimmunol.181.2.1025

http://www.jimmunol.org/content/181/2/1025

---

**Why The JI?**

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

---

**References**
This article **cites 48 articles**, 20 of which you can access for free at:
http://www.jimmunol.org/content/181/2/1025.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
FOXP3 Induced by CD28/B7 Interaction Regulates CD25 and Anergic Phenotype in Human CD4+CD25− T Lymphocytes

Cristiano Scottà, Marzia Soligo, Cristina Camperio, and Enza Piccolella

Among the signals necessary to generate CD4+CD25+FOXP3+ T cells from CD4+CD25−FOXP3− T cells, a pivotal role is played by CD28. However, in humans, it is not known whether CD28 signaling independently of TCR promotes forkhead box protein 3 (FOXP3) expression and regulates CD4+CD25+FOXP3+ T cell functions. To address this issue, starting from our previous experience, we analyzed the unique signals delivered by CD28 following stimulation by its natural ligand B7. Our results show that, in primary CD4+CD25− T cells, CD28 signals independent of TCR-mediated stimulatory pathways are sufficient to induce the transcription of FOXP3 in a small number of CD4+CD25− T cells committed to express FOXP3. These signals are dependent on CD28-derived PI3K/Akt pathways and resistant to cyclosporin A. In addition, we demonstrated that translated FOXP3 was the transcription of FOXP3 induced by CD28/B7 interaction regulates CD25 and repressor (21). Lack of FOXP3 leads to development of autoimmune disease by altering T cell function and can induce cytoskeleton rearrangements and up-regulate IL-2/4 independently of TCR (3, 4). We have shown that CD28 engagement by its ligand B7 can generate TCR-independent signals leading to IκB kinase complex and NF-κB activation (5, 6). Furthermore, CD28 engagement by B7 induces the transcriptional activation of NF-κB, Bcl-xL, and BAFF genes through the recruitment of RelA and p52 on their respective promoters (7). Second, perhaps the most paradoxical novel function of CD28 is its involvement in the development and homeostasis of CD4+CD25+ T cells known as regulatory T cells (Treg) (3). Treg are negative regulators of T cell signaling which contribute to T cell “anergy” and to maintenance of self-tolerance (8–10). Efficient generation of Treg in the thymus requires CD28 (11, 12) and in periphery its ligand B7 concurs to limit T cell activation by sustaining a population of CD4+CD25+ Treg (13). This supports the hypothesis that the presence of B7 in a mouse, even on cells that are not displaying the cognate Ag, controls adaptive immune responses (14). It is also clear that CD28 deficiency can lead to a reduced disease potential as well as to enhanced susceptibility to autoimmune disease by altering T cell effector and Treg compartments (11, 13, 15). Furthermore, CD4+CD25− T cells can be converted in vivo to become CD4+CD25+ T cells with regulatory function only in the presence of B7 (16). The importance of CD28 signaling in maturation and proliferation of Treg has been also characterized in vitro and both mouse and human Treg can be expanded using anti-CD3 and anti-CD28 Abs in the presence of high concentrations of IL-2 (13, 17, 18).

The FOXP3 gene, that encodes the forkhead box protein 3 (FOXP3) transcription factor, is thought to be the “master gene” of Treg (19, 20). FOXP3 acts as both a gene transcriptional activator and repressor (21). Lack of FOXP3 leads to development of autoimmune lymphoproliferative diseases and ectopic FOXP3 expression can phenotypically convert effector T cells to Treg cells (22). However, FOXP3 expression does not perfectly correlate with Treg suppressive function (23). Several lines of evidence suggest that CD28 is required for FOXP3 induction. It has been shown that CD28-mediated signals induce developing thymocytes to express FOXP3 and to initiate the Treg cell differentiation program (12); CD28 is also primarily required for the survival/homeostasis of TGF-β-converted thymic CD4+CD25+ Treg (24). It is widely accepted that FOXP3 expression in TCR-activated CD4+CD25+ T cells requires costimulation by CD28, also in humans (18, 23, 25–27).

Altogether the above reported data strongly suggest that CD28 is capable of delivering signals that promote CD4+CD25+FOXP3+ T cells. However, for several reasons such
as: 1) the complexity of signals that in vivo regulate FOXP3 expression, 2) the costimulatory nature of CD28 that amplifies TCR signaling, and 3) the difficulty to analyze in vitro the unique signal transduction pathway that specifically targets FOXP3 promoter, the effects of CD28 signaling independent of TCR-mediated signals in CD4+CD25+ T cells are still lacking. We explored this mechanism through which CD28 may regulate peripheral tolerance.

Materials and Methods

Cell lines and reagents

Murine L cells (Dap3) and murine L cells expressing human B7.1 (Dap3/B7) were cultured in complete DMEM (Life Technologies) supplemented with 50 μg/ml hygromycin B (Sigma-Aldrich). Inhibitors used were N-p-tosyl-l-phenylalanine chloromethyl ketone (TPCK, Sigma-Aldrich), 2-(4-morpholinoethyl)-8-phenoxy-1-benzopyran-4-one (LY294002; Calbiochem), 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2; Calbiochem), and cyclosporin A (CsA; Sigma-Aldrich). Anti-CD3 (X-35) and anti-CD28 (93/11001) were previously fixed for 30 min at room temperature with 0.05% glutaraldehyde.

Purification of CD4+CD25+ and CD4+CD25− T cells

Human peripheral blood was obtained from a healthy donor blood bank and PBMC were prepared by centrifugation with 106/ml either alone or in combination with fresh CD4+CD25+ T cells. Cells (2 × 105) were surface stained with FITC-conjugated anti-human CD4 (MEM-241) and anti-human CD25 (MEM-181) were obtained from Immunotech. rIL-2 was purchased from Roche.

CD28-mediated CD4+CD25− T cell activation

CD4+CD25− T cells (2 × 105/ml) were activated either with Dap3 (used as control) or Dap3/B7 as previously described (7). Dap3 and Dap3/B7 were previously fixed for 30 min at room temperature with 0.05% glutaraldehyde on culture plate. Alternatively, CD4+CD25+ T cells were activated with 1 μg/ml anti-CD28 cross-linked with 10 μg/ml goat anti-mouse.

Quantitative real-time PCR

Total RNA was extracted using TRizol reagent (Invitrogen) from 3 × 10⁶ purified T cells and was reverse-transcribed into cDNA by using Moloney murine leukemia virus reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed on an Applied Biosystems PRISM 7300 detection system. FOXP3 and CD25 message expression was determined by the TaqMan method of real-time PCR with GAPDH as endogenous control. TaqMan Universal PCR Master Mix and the FOXP3 primer/probe set (part no. Hs00203958_m1), the CD25 primer/probe set (part no. Hs00907778_m1), and the GAPDH primer/probe set (part no. Hs9999905_m1) were purchased directly from Applied Biosystems. Relative quantification was performed using the comparative cycle threshold method as described in User Bulletin edited by Applied Biosystems.

ChIP assay

ChIP assays were performed as previously described (29). Briefly, after fixing in 1% formaldehyde, 10 × 10⁶ cells were lysed for 5 min in 50 mM Tris (pH 8.0), 2 mM EDTA, 0.1% Nonidet P-40, and 10% glycerol supplemented with protease inhibitors. Nuclei were resuspended in 50 mM Tris (pH 8.0), 1% SDS, and 5 mM EDTA. Chromatin was sheared by sonication, centrifuged, and diluted 10 times in 50 mM Tris (pH 8.0), 0.5% Nonidet P-40, 0.2 M NaCl, and 0.5 mM EDTA. After preclearing with a 50% suspension salmon sperm (Sigma-Aldrich)-saturated protein A (Amersham Biosciences), lysates were incubated at 4°C overnight with anti-FOXP3 Ab (eBioscience). Immune complexes were collected with sperm-saturated protein A, washed three times with high salt buffer (20 mM Tris (pH 8.0), 0.1% SDS, 1% Nonidet P-40, 2 mM EDTA, and 500 mM NaCl) and five times with 1× Tris/EDTA. Immune complexes were extracted in 1× Tris/EDTA containing 1% SDS and protein-DNA cross-links were reverted by heating at 65°C overnight. DNA was extracted by phenol-chloroform and 1/10 of the immunoprecipitated DNA was used in each PCR. PCR was conducted in an automated DNA Thermal Cycler GeneAmp System 2400 (Applied Biosystems). The primers used were as follows: CD5, 5’-CCAGCCCCACACCTCCAGCAA-3’ and 5’-CC TCTTTTGGATGCGCGCCG-3’; CTLA-4, 5’-CCACCTGATTTCCA GATCC-3’ and 5’-AGGCGAGCGATCGTCTT-3’; and IL-2, 5’-CT CTACGATGATGCGCCTCT-3’ and 5’-TGTAAGATGCTATTGA GGGTGACACT-3’.

Immunoblotting

Protein extracts were obtained by lysing 10⁶ cells for 30 min at 4°C in lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1% Nonidet P-40) in the presence of protease and phosphatase inhibitors. Proteins were resolved by 10% SDS-PAGE and blotted onto nitrocellulose membranes. Blots were incubated with anti-FOXP3 (H-190) and anti-a-tubulin were purchased from Santa Cruz Technology. PE-conjugated anti-human FOXP3 Abs were either PCH101 and rat IgG2a isotype control were purchased from eBioscience or 259D/C7 and mouse IgG1 isotype control were purchased from BD Pharmingen.

Immunofluorescent staining and flow cytometry analysis

Cells (2 × 10⁵) were first surface stained with FITC-conjugated anti-CD4 or FITC-conjugated anti-CD25 Abs; after fixation and permeabilization, cells were incubated with PE-conjugated anti-human FOXP3 Abs or FITC-conjugated anti-CD25 Abs or isotype controls according to the manufacturer’s instructions. BD Biosciences. Cytofluorometric bead array human cytokine kit was used to measure IL-2 levels. Data acquisition and analysis were conducted on a flow cytometric platform using BD Bioscience CellQuest and cytofluorometric bead array software.

Detection of apoptotic T cells

Apoptosis was measured by staining with FITC-conjugated annexin V and propidium iodide according to the manufacturer’s instructions. Flow cytometric analysis was performed on a BD Biosciences FACSCalibur.

Proliferation assay

Proliferation assays were performed on CD4+CD25− T cells stimulated or not with anti-CD28. CD4+CD25− and CD4+CD25+ T cells were plated at 5 × 10⁴ cells/well in 96-well plates with 5 × 10⁴ cells/well CD4−, depleted PBMC as feeders in the presence or absence of 40 IU/ml IL-2 and activated with anti-CD3 and anti-CD28 Abs. Cells were pulsed with 1 μCi/well [3H]thymidine on day 3 and proliferation was assessed 18 h later using a liquid scintillation counter.

Suppression assay

Fresh autologous CD4+CD25+ responder T cells were stimulated with anti-CD3 and anti-CD28 Abs in the presence of 5 × 10⁴ feeders cells/well and plated at 5 × 10⁴/well either alone or in combination with fresh CD4+CD25+ T cells or in combination with CD28-activated CD4+CD25−
FIGURE 1. FOXP3 is up-regulated in CD4⁺CD25⁻ T cells by CD28 stimulation. Human primary CD4⁺CD25⁻ T cells were stimulated with adherent Dap3/B7 cells for different times. As controls, freshly isolated CD4⁺CD25⁻ T cells (CD25⁻) have been included in the experiments. A, FOXP3 mRNA level was measured by real-time PCR. B, CD4⁺CD25⁻ T cells were pretreated for 2 h with 25 μM TPCK, 50 μM LY294002, 50 μM PP2, and 1 μg/ml CsA and then stimulated for 12 h with adherent Dap3/B7 cells. FOXP3 mRNA expression was analyzed by real-time PCR. Bars show the mean ± SD of one representative experiment of five performed. FACS analysis was performed both on CD25⁺ and resting CD25⁻ (t₀), and after 48 h (t₄₈) on Dap3- and Dap3/B7-stimulated CD25⁻ cells stained with PE-conjugated anti-FOXP3 Ab (PCH101) to evaluate intracytoplasmic FOXP3 expression reported as MFI and percent positive cells of one representative donor (C) and five independent experiments (D). Total extracts from CD4⁺CD25⁻ T cells isolated from two different donors, stimulated with adherent Dap3/B7 cells for different times, were analyzed by Western blotting with anti-FOXP3 Ab. Total extract from CD4⁺CD25⁻ T cells isolated from the same donor was also analyzed (E). The blots were reprobed with anti-α-tubulin Ab to verify equal loading of proteins.

Results

FOXP3 and CD25 are up-regulated in CD4⁺CD25⁻ T cells by CD28

To study the effect of CD28 signaling on FOXP3 expression, we analyzed the interaction of CD28, expressed on CD4⁺CD25⁻ T cells, with its natural ligand B7. To this purpose, we measured cell growth, FOXP3 mRNA levels, and FOXP3 protein expression (Fig. 1) in CD4⁺CD25⁻ T cells cultured with Dap3 or Dap3/B7 murine fibroblasts. The levels of FOXP3 mRNA and protein in freshly isolated CD4⁺CD25⁻ T cells were also determined. A time course of FOXP3 mRNA quantification in CD4⁺CD25⁻ T cells, representative of five performed experiments with different donors, is reported in Fig. 1A. The shown histogram describes a classical Gaussian-like shape with a maximum of FOXP3 mRNA synthesis at 12 h. However, depending on the donor, maximum synthesis of FOXP3 mRNA could also occur at 24 and 48 h (data not shown). Fig. 1E shows the expression of FOXP3 protein in two different donors, who reached FOXP3 maximum synthesis at 24 and 48 h after stimulation, respectively. FACS analysis of intracellular FOXP3 stained with anti-FOXP3 mAb PCH101 (Fig. 1, C and D), performed to analyze FOXP3 expression at the single-cell level, shows an increase in the frequency of CD4⁺FOXP3⁺ T cells such as mean fluorescence intensity (MFI) after 48 h of culture in the presence of Dap3/B7, and ~20% (range, 5–40%) of CD28-activated cells stained positive for FOXP3. On the contrary, no increase of intracellular FOXP3 was observed in cultures without B7. To exclude that CD28-activated cells were stained nonspecifically with PCH101, 259D/C7 mAb was also used (30); T cells stained in parallel with PCH101 and 259D/C7 gave similar results (data not shown). Although at t₀ FOXP3 was already present in ~4% (range, 0.5–5%), the possibility that CD28-activated FOXP3⁺ T cells expanded from the unstimulated FOXP3⁺ T cells is quite impossible in the absence of cell division. Indeed, CFSE-labeled CD4⁺CD25⁻ T cell measures demonstrated that the interaction of CD28 costimulatory molecules with B7 did not influence T cell division (data not shown). Moreover, unstimulated CD4⁺CD25⁻ T cells expressed even higher levels of FOXP3 in comparison to CD28-activated CD4⁺CD25⁻ T cells (Fig. 1, A and C). This suggests that after CD28 signaling CD4⁺FOXP3⁺ T cells predominantly arose from CD4⁺FOXP3⁻ T cells, although with levels of FOXP3 comparable to that observed in CD4⁺CD25⁻ T cells lacking Treg function (27). To characterize the CD28-mediated biochemical pathways involved in FOXP3 transcription, we used different inhibitors. The results in Fig. 1B show that LY294002, a specific inhibitor of PI3K/Akt, and TPCK, an NF-κB inhibitor, significantly inhibited FOXP3 mRNA synthesis, consistent with the requirement of PI3K/Akt in transducing the CD28 signal and favoring NF-κB translocation to the nucleus. On the contrary, PP2, an inhibitor of src tyrosine kinases, and CsA, an inhibitor of calcineurin, did not show any activity on FOXP3 transcription, confirming the resistance of the CD28 pathway to CsA
Altogether these results strongly support that a unique signal mediated by CD28 may activate FOXP3 transcription and translation. The influence of TGF-β on FOXP3 expression, known to be present in human serum, has been excluded since the stimulation of CD4⁺CD25⁻ T cells for 48 h with Dap3/B7 in serum-free synthetic medium gave results similar to those obtained with human serum (data not shown).

It is common opinion that agonistic Abs do not always mimic the triggering signals mediated by natural ligands. Discordant results may be obtained depending on many conditions such as Ab concentration, affinity for the recognized epitopes, and position of the binding site on the target molecules (32). However, most of the published results on CD28 signaling have been obtained with mAbs. Therefore, to compare the effect of the natural ligand B7 with that obtained by mAbs, we analyzed in time course experiments the effect of cross-linked anti-CD28 mAb on FOXP3 expression in CD4⁺CD25⁻ T cells and in parallel the effects of anti-CD3 and anti-CD3 plus anti-CD28 Abs ( Fig. 2 ) for the indicated times. Cell lysates were analyzed by SDS-PAGE and blotted with anti-FOXP3 Ab. Densitometric analysis for expression of normalized FOXP3 is shown in A. Western blotting images of 12-h cell lysates reported in A are shown in B. As control, CD4⁺CD25⁻ T cell lysates from the same donor are shown. CD4⁺CD25⁻ T cells, stimulated with anti-CD28 (C) or anti-CD3 plus anti-CD28 Abs (D) for the indicated times, were treated with CsA (1 µg/ml) and were analyzed by Western blotting. Results are representative of three independent experiments.

![FIGURE 2.](https://example.com/figure2.png)

**FIGURE 2.** FOXP3 is up-regulated, with different kinetics, in CD4⁺CD25⁻ T cells stimulated with anti-CD3, anti-CD28, and anti-CD3 plus anti-CD28 mAbs. CD4⁺CD25⁻ T cells were stimulated with anti-CD3, anti-CD28, and anti-CD3 plus anti-CD28 Abs for the indicated times. Cell lysates were analyzed by SDS-PAGE and blotted with anti-FOXP3 Ab. Densitometric analysis for expression of normalized FOXP3 is shown in A. Western blotting images of 12-h cell lysates reported in A are shown in B. As control, CD4⁺CD25⁻ T cell lysates from the same donor are shown. CD4⁺CD25⁻ T cells, stimulated with anti-CD28 (C) or anti-CD3 plus anti-CD28 Abs (D) for the indicated times, were treated with CsA (1 µg/ml) and were analyzed by Western blotting. Results are representative of three independent experiments.

CD28 signaling has been described to be CsA resistant or sensible depending on the absence or presence of TCR ligation, respectively (35), we tested whether the levels of FOXP3 protein induced by anti-CD28 and anti-CD3 plus anti-CD28 could be

![FIGURE 3.](https://example.com/figure3.png)

**FIGURE 3.** CD25 is up-regulated by CD28 stimulation. CD4⁺CD25⁻ T cells were stimulated with adherent Dap3/B7 cells for different times. A, The CD25 mRNA level was measured by real-time PCR. B, CD4⁺CD25⁻ T cells were pretreated or not with inhibitors (as described in Fig. 1B). Bars show the mean ± SD of one representative of five performed experiments. C, FACS analysis was performed on resting (t₀) or activated (t₄₈) CD4⁺CD25⁻ T cells stained with FITC-conjugated anti-CD25 mAb to evaluate membrane CD25 expression reported as MFI and percent positive cells.
similarly affected by CsA. This is an important analysis for at least two different reasons: 1) we have shown that FOXP3 transcription induced by B7.1 was completely CsA resistant (Fig. 1B) but we do not know the sensitivity to CsA of FOXP3 induced by cross-linked anti-CD28 mAb and 2) we can compare our experiments with those performed using TCR plus CD28 that reported the sensitivity of FOXP3 to CsA (25). Therefore, we measured FOXP3 expression in anti-CD28 and anti-CD3 plus anti-CD28 T cell cultures activated for 12 and 48 h in the presence or absence of CsA (Fig. 2, C and D). Interestingly, although FOXP3 in anti-CD28 cultures were CsA resistant at both time points, FOXP3 expression in anti-CD3 plus anti-CD28 cultures was CsA resistant at 12 h and CsA sensitive at 48 h, suggesting that CD28 and TCR influenced FOXP3 expression by different pathways.

Recent evidences support that FOXP3 regulates the expression of CD25 in mice (36). To verify this, we performed a set of experiments similar to those described in Fig. 1. It was interesting to observe, in Fig. 3A, that the profile of the histogram of CD25 mRNA expression is completely different from that of FOXP3, in that the maximum synthesis of CD25 was delayed with respect to FOXP3. Moreover, the addition of FOXP3 mRNA inhibitors LY294002 and TPCK strongly suppressed CD25 expression. PP2 gave a less marked effect (Fig. 3B). Altogether these data support the role of FOXP3 in the transcriptional regulation of CD25 and suggest that the pathway CD28/PI3K/NF-κB is deeply involved in the transcriptional activation of FOXP3 and consequently CD25. The analysis of the frequency and MFI of CD4+CD25+ FOXP3+ T cells at t₀ and t₄₈ confirms that FOXP3 expression correlates with cell surface CD25 expression (Fig. 3C).

**IL-2 rescues CD4+CD25+ FOXP3+ T cells from apoptosis but does not influence FOXP3+ and CD25+ expression**

Although time course experiments showed a decrease in FOXP3 expression after 48 h from the beginning of CD28 stimulation, we could not analyze its expression at later times because CD28-activated CD4+ CD25+ T cells were stimulated with adherent Dap3/B7 cells for different times with or without 20 IU/ml (A) and 100 IU/ml (B) of IL-2. FOXP3 protein levels were subsequently assessed by Western blotting. Data represent one of three independent experiments. C, CD25 expression was measured by flow cytometry and reported as percentage of positive cells in the absence (0–48 h) or presence of rIL-2 (72 h).

**FIGURE 4.** IL-2 protects CD4+ CD25+ T cells from CD28-induced apoptosis. Apoptosis of CD4+CD25+ T cells was analyzed by FACS before and after 72 h of stimulation with adherent Dap3 (control) or Dap3/B7 cells in the absence (B) or presence (C) of 20 IU/ml rIL-2 by staining with FITC-conjugated annexin V+ and propidium iodide. D, Bars represent the percentage of apoptosis measured at 48 and 72 h in the presence of different rIL-2 doses in control (Ctr) and stimulated (Dap3/B7) cultures.
CD4+CD25+ T cells were stimulated with adherent Dap3/B7 cells for different times. ChIP assays were performed by using anti-FOXP3 Ab or no Ab. Immunoprecipitated DNA was analyzed by PCR with CD25 (A), CTLA-4 (B), and IL-2 (C) promoter-specific primers. D, CD4+CD25+ T cells were activated for 24 h with anti-CD28 and treated or not with CsA. FOXP3 binding to IL-2 promoter was analyzed by ChIP assay.

CD4+CD25+ T cells became susceptible to apoptosis. Indeed, Fig. 4, A and B, shows the increase of annexin V+ stained T cells in CD28-activated CD4+CD25+ T cells cultured for 72 h with Dap3/B7 cells. However, addition of exogenous IL-2 significantly prevented apoptosis (Fig. 4C) in a dose-dependent manner (Fig. 4D). The evidence that IL-2 favored T cell viability suggested to us to measure FOXP3 and CD25 following the event described as typical of Treg, suggested to us to verify whether CD28 stimulation of human CD4+CD25+ T cells induces the selective recruitment of FOXP3 to the CD25, Ctla4, and Il-2 promoters. CD4+CD25+ T cells were stimulated with adherent Dap3/B7 cells for different times. ChIP assays were performed by using anti-FOXP3 Ab or no Ab. Immunoprecipitated DNA was analyzed by PCR with CD25 (A), CTLA-4 (B), and IL-2 (C) promoter-specific primers. D, CD4+CD25+ T cells were activated for 24 h with anti-CD28 and treated or not with CsA. FOXP3 binding to IL-2 promoter was analyzed by ChIP assay.

It has been shown by ChIP experiments that FOXP3 could occupy CD25, Ctla4, and Il-2 promoters in both murine CD4+CD25+ Treg cells and Jurkat T cells retrovirally transduced with FOXP3 and stimulated by TCR plus CD28 and/or ionomycin (37). We verified this phenomenon in primary CD4+CD25+ T cells activated for 24 and 48 h by Dap3/B7. The results reported in Fig. 6 show for the first time, to the best of our knowledge, that the activatory pathways mediated by CD28 in CD4+CD25+ T cells favor not only the transcription and translation of FOXP3, but also its occupancy of CD25, Il-2, and Ctla4 promoters. Interestingly, after 24 h, FOXP3 was recruited on all studied promoters, but the occupancy of CD25 and Il-2 promoters (Fig. 6, A and C) was maintained for all of the observation time, whereas FOXP3 exited the Ctla4 promoter between 24 and 48 h from the activation (Fig. 6B). To analyze the sensitivity of FOXP3 recruitment on Il-2 promoter to CsA, we stimulated CD4+CD25+ T cells with anti-CD28 mAbs for 24 h in the presence and absence of CsA (Fig. 6D). CsA induced a slight decrease of FOXP3 recruitment on the Il-2 promoter, suggesting that, although CD28 signaling regulates FOXP3 expression in a CsA-resistant manner, signals from calcineurin may have a role in this system (38).

CD4+CD25+ T cells were analyzed by flow cytometry after 3 days of culture. A, Cell division profiles by CFSE labeling of anti-CD3 plus anti-CD28 CD4+CD25+ responder T cells (T) alone or cocultured in the presence of a 1:1 ratio of either autologous CD4+CD25+ (TCD25) or CD4+CD25+ T cells (TCD25) were analyzed by flow cytometry after 3 days of culture. B and C, CD4+CD25+ T cells (TCD25i) purified from CD28-activated CD4+CD25+ T cells after 48 h of culture and CD4+CD25− control T cells (T) were stimulated with anti-CD3 plus anti-CD28 in the presence or absence of rIL-2 (40 IU/ml). Proliferative responses of CD4+CD25+ T cells stimulated immediately after purification (B) or after 7 days in IL-2 culture medium (C) were determined by [3H]thymidine incorporation. Results are expressed as means ± SD of triplicate cultures. The upper right inset of B shows IL-2 release in culture medium (sample) after 48 h by T cells used in the stimulation assays. Data are representative of three different donors.
CD28-activated T cells expressing FOXP3 and CD25 had acquired suppressive activity. Therefore, CD28-activated or CD4+CD25−unstimulated T cells (natural Treg), purified using a Treg cell isolation kit, were added to autologous CD4+CD25+ T cells (responder cells) and cultured in anti-CD3-coated wells with anti-CD28 for 5 days. Fig. 7A, where the effects of CD28 on cell growth of responder cells labeled with CFSE were analyzed, shows that CD28-activated CD4+CD25+ failed to mediate suppressive activity while autologous natural Treg, used as control, induced a dramatic inhibition of cell growth. It has been described that the transient expression of FOXP3 in anti-CD3-activated non-regulatory CD4+ T cells is strongly associated with hyporesponsiveness (27). We investigated whether this could also occur after CD28 activation. Therefore, we purified CD4+CD25+ T cells from CD28-activated CD4+CD25+ T cells after 48 h of culture. These cells were either immediately stimulated or allowed to rest in IL-2 culture medium for 7 days until FOXP3 was completely down-regulated and stimulated. The release of IL-2 in the culture medium and the effect of exogenous IL-2 have been also tested. Fig. 7B shows that CD4+CD25+ T cells purified from CD28-activated CD4+CD25+ T cells and expressing FOXP3 are completely unresponsive to mitogenic stimuli. Indeed, these cells were unable to proliferate and release IL-2 (inset, Fig. 7B). On the contrary, the same cells, which become FOXP3+ after 7 days in IL-2 culture medium (data not shown), were similar to the non-CD28-activated CD4+CD25+ T cells in that they proliferated when stimulated (Fig. 7C).

Discussion
Both in mice and in humans CD4+CD25+ T cells with regulatory functions are important components of peripheral tolerance that is responsible in the control of autoreactive T cells in normal individuals. FOXP3 transcription factor is a critical regulator of these cells (22). Among the signals necessary to generate CD4+CD25+FOXP3+ T cells, a pivotal role is played by CD28. However, while in mice it was possible to study the role played by the interaction of CD28 with its ligand B7 in the activation and maintenance of CD4+CD25+FOXP3+ T cells, in humans similar results are lacking. Taking advantage from our previous results that allowed us to characterize a human model system for the analysis of the CD28/B7-dependent pathways in T cells, we have approached two important issues: the effect of the unique signal delivered by CD28 in CD4+CD25−T cells and how FOXP3 influences the phenotypic and functional properties of CD28-activated CD4+CD25−T cells.

The TCR and CD28 are independent signaling units and a TCR-mediated FOXP3 expression. One of the hallmarks of CD28 stimulation is its insensitivity to the immunosuppressive drugs CsA and FK506; therefore, the lack of effect of CsA on CD28-mediated activation of FOXP3 is consistent with previously published reports (31, 43). The differential sensitivity to calcineurin inhibitors of CD28 signal triggered alone or in association with TCR has been also demonstrated in genomic expression programs (35). However, although CsA is an inhibitor of the calcineurin/NFAT pathway, the CsA-resistant CD28 signal has been described to result in the activation and binding of NFAT to DNA (44) and in enhancing nuclear occupancy of NFAT proteins by CD28-mediated inhibition of GSK3 (35, 45). Since many experimental evidences support the need of NFAT for favoring either the transcription of FOXP3 or its binding to DNA (25, 37, 38), the binding of NFAT to FOXP3 and Il-2 genes in CD28-activated CD4+CD25−T cells cannot be excluded. This idea is currently being tested.

Downstream of PI3K/Akt, the effects of the CD28 unique signal include the activation of NF-κB (7), indicating a possible role for NF-κB in FOXP3 expression in CD4+CD25−T cells. It has been described that FOXP3 physically associates with NF-κB and blocks its ability to induce the expression of a NF-κB-dependent gene (46), but a role of NF-κB in FOXP3 expression is lacking. The observation that inhibitors of NF-κB suppress FOXP3 expression and specific NF-κB units are recruited on the FOXP3 promoter (M. Soligo, C. Camperio, C. Scottà, and E. Piccolenda, manuscript in preparation) would be consistent with the view that CD28-mediated activation of NF-κB may represent another decisive signal.

It has been reported that mature peripheral CD4+CD25−T cells can convert to CD4+CD25+ Treg cells spontaneously in vivo in a thymus-independent but B7-dependent manner (16). The converted cells failed to proliferate after stimulation and expressed high levels of FOXP3 mRNA. Our data allow us to speculate that
this may also occur in humans, although in a transient way. Indeed, we have demonstrated that CD28-mediated activation of FOXP3 and its recruitment on CD25, II-2, and CD40L promoters converted a small subpopulation of CD4+CD25- T cells to CD4+CD25+ T cells and acquired an anergic phenotype. Indeed, in these cells, T cell proliferation and IL-2 synthesis were impaired. However, we present evidence that this unresponsiveness was not maintained in the absence of FOXP3, and T cell proliferation could be easily reconstituted upon subsequent exposures to Ag. This suggests that the induction of FOXP3, at least in a small number of CD4+CD25- T cells committed to express FOXP3, in the absence of TCR signaling, represents a new mechanism by which FOXP3 can mediate a transient shut down of the TCR pathways. We have also shown that the delivery of the CD28 signal in CD4+CD25- T cells led to the propensity for apoptosis. However, the evidence that exogenous IL-2 rescued CD4+CD25+ T cells from apoptosis but did not affect the decrease of FOXP3 and CD25 supports the view that FOXP3-driven enhancement of CD25 is important for T cell survival (47, 48).

In conclusion, we have not only confirmed in humans that CD28 provides an unique signal to promote FOXP3 expression necessary to convert human CD4+CD25- T cells to CD4+CD25+ T cells, but we went on demonstrating that FOXP3 up-regulation occurred independently of TCR. This suggests a scenario where the expression of B7 on professional APCs may contribute significantly to the homeostasis of CD4+CD25- T cells expressing or committed to acquire FOXP3 and to induce in these cells a transient non-responsiveness important for the maintenance of peripheral tolerance.

Acknowledgments

We thank the unknown reviewers of this manuscript for their constructive comments. We thank Paola Del Porto for useful suggestions and Antonio Costanzo for helpful discussion and critical reading of this manuscript.

Disclosures

The authors have no financial conflict of interest.

References


11. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell de-


19. Tran, D. Q., H. Ramsay, and E. M. Shevach. 2007. Induction of Foxp3 expres-

sion in naive human CD4+CD25+ T cells by T-cell receptor stimulation is trans-


Raf-1/MEK-1/ERK-2-independent pathway in stimulation of IL-2 gene transcrip-


