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CD28 costimulation regulates FOXP3 in a RelA/NF-κB-dependent mechanism

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The molecular mechanisms whereby CD28 alone or associated with TCR can regulate FOXP3 expression are not understood, although the importance of CD28 as a pivotal regulator of CD4$^+$CD25$^+$FOXP3$^+$ T cells is well recognized. We previously demonstrated that unique CD28-induced, NF-κB-dependent signals were sufficient to activate FOXP3 transcription in human CD4$^+$CD25$^-$ T cells; however, the exact mechanisms are currently unknown. In this study, we have identified novel κB-binding sites on *FOXP3* gene and demonstrated that CD28 signals mediated FOXP3 trans activation by nuclear translocation of RelA/NF-κB and not of c-Rel. The occupancy of FOXP3 κB-binding sites by RelA dimers that correlated with histone acetylation and recruitment of Pol II were required both to initiate FOXP3 transcription and to control the promoter occupancy by NFAT. Interestingly, knockdown of RelA in CD4$^+$CD25$^-$ T cells stimulated through TCR and CD28 significantly affected FOXP3 expression, confirming that also the transcriptional activation of *FOXP3* gene by TCR in the presence of CD28-costimulatory signals is RelA-dependent. In conclusion, these data suggest a new mechanism by which FOXP3 is activated and supports the critical role of CD28 in the regulation of peripheral tolerance.

Key words: CD28 · FOXP3 · RelA/NF-κB · T-cell activation

Supporting Information available online

Introduction

The CD4$^+$CD25$^+$FOXP3$^+$ regulatory T cells (Treg) are a specialized subset of CD4$^+$ T cells that can suppress the responses of effector T cells. Treg maintain peripheral immune tolerance and prevent chronic inflammation as well as autoimmune diseases [1]. Treg are characterized by the expression of the α-chain of the interleukin-2 receptor (CD25) and by the transcription factor forkhead box P3 (FOXP3). This factor is not only a marker for Treg, but also programs their regulatory functions [2]. The majority of Treg are derived from the thymus, natural Treg, although a small percentage of peripheral CD4$^+$CD25$^-$ T cell could be converted into FOXP3-expressing Treg (induced Treg (iTreg)) in pathologic inflammatory conditions [3]. Peripheral

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commitment of CD4$^+$CD25$^-$ T cell is controlled by signaling through T-cell antigen receptor (TCR) and accessory molecules such as TGF-β1 and IL-2 [4]. All this is necessary but does not induce maximal FOXP3 expression as it is observed in natural Treg. In fact costimulation through the interaction of CD28 with its ligand B7 is strictly required for the activation of FOXP3 and for the maturation and differentiation of Treg either in vivo or in vitro [5, 6]. However, the molecular mechanisms of CD28-mediated FOXP3 induction remain unknown.

Accumulating evidence suggests that the transcription factor NF-κB in T lymphocytes is the most relevant target downstream of CD28 biochemical pathways [7]. NF-κB can be composed as a combination of five related proteins, RelA/p65, c-Rel, RelB, p50 and p52 that form homo- and hetero-dimeric complexes [8]. Many reports demonstrate that NF-κB constitutes an essential signal for Treg development and homeostasis in vivo [9, 10]. More recently, c-Rel has been identified as the major NF-κB factor that initiates FOXP3 transcription in thymic Treg precursors [11–14]. The role of c-Rel in peripheral Treg generation is less clear. Indeed, despite a significant reduction in number of peripheral Treg in c-rel$^{-/-}$ mice, the residual c-rel$^{-/-}$ Tregs express normal levels of FOXP3 and exhibit a phenotype similar to that of normal Treg [12]. Therefore, the iTreg could be not absolutely dependent on c-Rel.

The *FOXP3* promoter is located 6.5 kb upstream of the first coding exon of FOXP3 [15] and its activation is induced by several transcription factors. In addition to TCR-induced NFAT and AP-1[15], two NF-κB-binding sites coincident with the previously designated NFAT sites in the human *FOXP3* promoter [15] have been revealed [12]. IL-2-induced STAT5 and IL-27-induced STAT1 directly bind to the promoter region [16, 17], and the same region can be also occupied by Smad and pCREB [12]. Moreover cooperation between TGF-β-inducible early gene 1 (TIEG1) and itchy E3 ubiquitin protein ligase homologue (ITCH) to induce FOXP3 expression has been also observed [18].

We have previously demonstrated that CD28 unique signals induced the expression of FOXP3 in human CD4$^+$CD25$^-$ T lymphocytes in a cyclosporine A (CsA)-independent manner [19]. Since CD28 activation leads to the preferential nuclear translocation of RelA, and not of c-Rel [20], we asked whether RelA could activate FOXP3 in iTreg. The current study reveals novel RelA-binding sites on *FOXP3* promoter and demonstrates that the recruitment of CD28-activated RelA on these sites is sufficient to mediate the trans activation of *FOXP3* gene. Thus CD28-induced RelA is a key transcription factor of FOXP3 expression in human CD4$^+$CD25$^-$ T cells.

Results

Identification of NF-κB-binding sites on FOXP3 promoter

The human *FOXP3* promoter, located −1657 bp upstream and +176 bp downstream of the transcription start site (TSS), has been previously cloned (construct −1657/+176). The promoter activity was characterized in primary CD4$^+$ T cells and NFAT and AP-1-binding sites have been characterized in the region between −511 and −348 [15]. To test the hypothesis that CD28-activated RelA could control human *FOXP3* gene expression, we have explored whether *FOXP3* −1657/+176 promoter construct presented NF-κB-binding motifs. The sequence analysis has identified four putative κB sites in the region between −936 and −646 and two more in the region between +121 and +148 (positions −936, −873, −758, −655, +121 and +139, Supporting Information Fig. 1). All these sites are 10-base pairs in length and show the conventional κB-binding site sequence [8]. Human and mouse promoter sequence comparison by m-Vista aligment (Supporting Information Fig. 1) shows that, despite the conservation of 87% between mice and human, only the sites at positions −873 and +139 conserved the binding motif described in mice [21]. To verify whether *FOXP3* promoter could be trans activated by RelA, HEK 293 cells were co-transfected with FOXP3 −1657/+176 construct and empty vector or with expression vector encoding RelA or with an RelA vector mutated on DNA-binding region, RelA YA ED [22]. After 24 h, luciferase reporter gene activity was measured and Fig. 1A shows a remarkable dose-dependent increase of luciferase activity with RelA that was abolished using the RelA YA ED. No activity was detected in HEK 293 cells transfected with the only FOXP3 −1657/+176 construct. To verify the degree to which the four putative κB sites upstream of Rel/NFAT sites [12, 15] could concur to mediate trans activating functions (Fig. 1B), we used 5′ deletions (−1210, −771, −465, −348, −211 and −90). The most prominent reporter activity was obtained with the construct −1210/+176, and the lost of two and four putative κB sites in the construct −771 and −465, respectively, showed a significant reduction of luciferase activity. The other constructs (−348, −211 and −90) did not show any significant increase of activity comparing to the control plasmid. The co-transfection of the construct −1210 and −465 into cell lines of lymphoid and non-lymphoid origin Jurkat, HeLa, HCT116 and NCI-H1299 cells with RelA gave similar results (Supporting Information Fig. 2). Therefore, the direct interaction of RelA homodimers with DNA is sufficient to trans activate FOXP3 construct, independently of cell specificity. We also verified whether the human constructs FOXP3 −1210/+176 and −465/+176 could be activated by c-Rel. Dose-response experiments were performed and the results are reported in the Supporting Information Fig. 3. The data show that c-Rel homodimers, differently of RelA (Fig. 1), activates at similar levels the human FOXP3 −1210/+176 and −465/+176 constructs, supporting the presence of Rel/NFAT-binding sites on the region −480 to −144 of human *FOXP3* promoter.

To determine the effect of the putative κB-binding sites in the region between −936 and −646 we performed site-directed mutagenesis of these sites. Five different constructs were generated as described in Supporting Information Table 1. The results shown in Fig. 1C demonstrate that the single mutations at positions −873, −758 and −655 reduced the induction of the reporter gene between 78 and 65% comparing to the WT, while

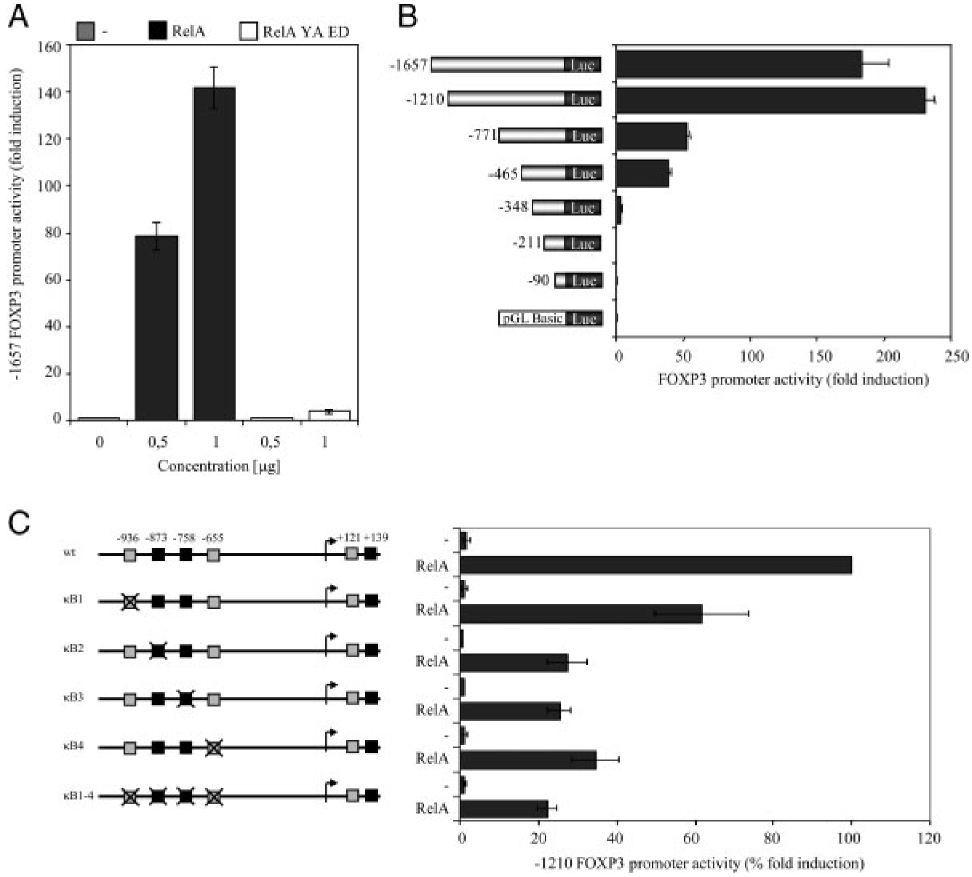

Figure 1. RelA/NF-κB binds *FOXP3* promoter in HEK 293 cells. (A) HEK 293 cells were transfected with FOXP3 −1657/+176 promoter luciferase reporter vector and, where indicated, were co-transfected with RelA or RelA YA ED, at different concentrations. (B) HEK 293 cells were transfected with pGL4 basic or vector containing different length of *FOXP3* promoter region and co-transfected with RelA. The results in (A) and (B) are calculated as luciferase units/protein concentration and represent one of five independent experiments performed in triplicate. Bars show the mean ± SD. (C) The human FOXP3 −1210/+176 promoter region containing the six putative NF-κB-binding sites was mutated in a site-directed manner. Five different constructs were generated. The luciferase reporter vectors, containing the mutated κB sites, were transfected into HEK 293 cells. Where indicated, the cells were co-transfected with RelA expression plasmid, and the luciferase activity was measured. The results are calculated as luciferase units/protein concentration. Data are expressed as fold induction relative to WT pGL4 FOXP3 −1210/+176 and represent one of five independent experiments. Effect of mutagenesis is shown as percentage relative to WT construct, co-transfected with RelA and arbitrarily set to 100%.

the mutation of the binding site at position −936 exhibited slighter inhibition of luciferase activity (38%).

RelA and NFAT cooperate to trans activate *FOXP3* promoter

It has been demonstrated that the activation of *FOXP3* gene expression in murine $CD4^{+}CD25^{-}$ T cells activated by Treg-inducing factors is dependent on the recruitment of c-Rel, RelA and NFATp on the *FOXP3* promoter region −501 to −239 [12]. Moreover, a human *FOXP3* promoter fragment (ECR1_525) could be transactivated by RelA, but not by NFATp [23]. This prompted us to investigate the effect of the co-expression of NFAT and RelA on the activation of human *FOXP3* promoter. HEK 293 cells were co-transfected with the construct −1210/+176 and RelA or NFATp expression vectors or with RelA and NFATp both in the absence and presence of Io (Fig. 2 and Supporting Information Fig. 4). RelA and NFATp protein levels were determined by immunoblotting (Supporting Information Fig. 5). Although RelA shows clear activatory properties (Fig. 2A), NFAT did not affect the activity of FOXP3 −1210/+176 construct in HEK 293 cells, despite the activation by Io (Supporting Information Fig. 4). However, when dose response experiments were performed, co-expression of NFAT and RelA in HEK 293 cells resulted in a synergistic effect on promoter activity (Fig. 2B). Indeed 240-fold (±78) increase in luciferase activity was measurable with 1 µg of RelA plus 3 µg of NFAT with respect to 1 µg RelA alone (120-fold ± 38, $p<0.05$), and 400-fold (±131) increase in luciferase activity was measurable with 2 µg of RelA plus 3 µg of NFAT with respect to 2 µg RelA alone (150-fold ± 47, $p<0.01$). To determine whether the recruitment of RelA on κB-binding sites could influence the occupancy of *FOXP3* promoter by NFAT, we used RelA YA ED and assayed its effect on the trans activation of FOXP3 reporter in the presence of NFAT

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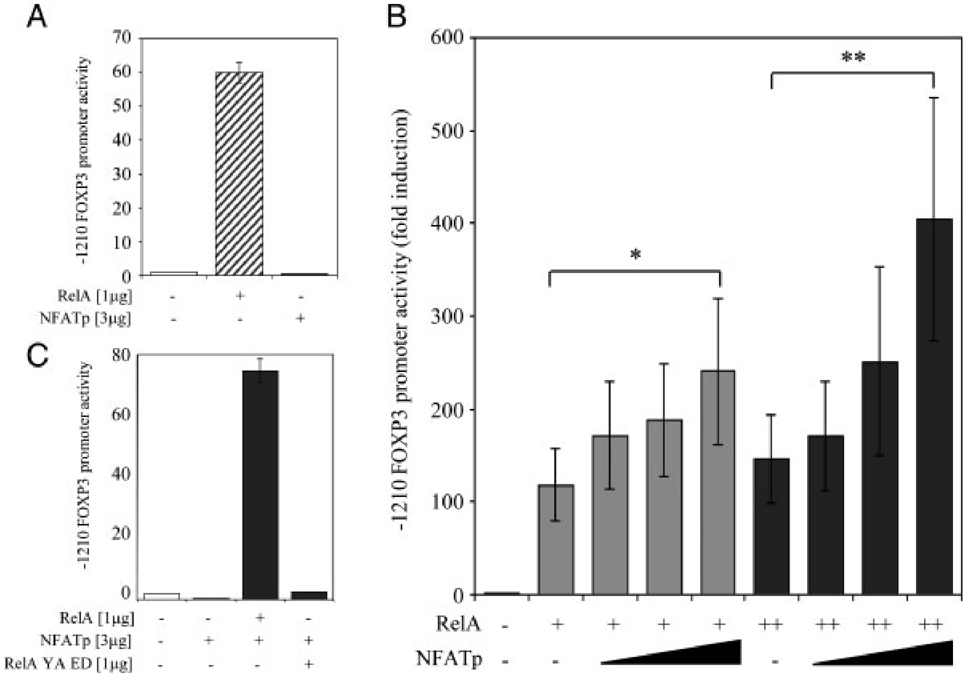

Figure 2. RelA and NFAT cooperate to trans activate *FOXP3* promoter. HEK 293 cells were transfected with FOXP3 -1210/+176 promoter luciferase reporter vector and co-transfected with RelA or NFAT (A). FOXP3 −1210/+176 transfected HEK 293 cells were co-transfected with RelA [1 μg (+), 2 μg (++)] plus NFAT (1–3 μg) expression plasmids (B). HEK 293 cells were transfected with FOXP3 −1210/+176 promoter luciferase reporter vector and co-transfected with RelA plus NFAT or RelA YA ED plus NFAT expression plasmids (C). The results are calculated as luciferase units/protein concentration and expressed as mean ± SD. (A) and (C) show one of five independent experiments performed in triplicate, while (B) shows the mean ± SD of five independent experiments. $^{*}p<0.05$, $^{**}p<0.01$ based on Student's two-tailed *t*-test.

(Fig. 2C). Notably, the RelA YA ED was completely unable to drive NFAT to trans activate *FOXP3* promoter.

Trans activation of *FOXP3* promoter in CD4$^+$CD25$^-$ T cells by CD28-signaling

We have previously demonstrated that the activation of CD28 pathways by B7 leads to the preferential nuclear translocation of RelA [20] and to the activation of FOXP3 transcription [19]. To verify whether the activation of endogenous RelA by CD28 could mediate *FOXP3* promoter activation, CD4$^+$CD25$^-$ T cells were transfected with the construct −1210/+176 and luciferase activity was measured after stimulation with Dap3/B7. Figure 3A shows that in CD28-activated T cells a threefold increase of luciferase activity was detected after 8 h of activation. The nuclear translocation of both RelA and c-Rel was also analyzed and Fig. 3B shows a significant increase of nuclear RelA at 2 h after stimulation, while c-Rel was not detected in the nucleus until 8 h of observation. Thus, endogenous RelA, recruited on construct −1210/+176 after CD28 engagement could be responsible of the increase of luciferase activity. To verify whether the mutations of κB-binding sites could modify the reporter activity, the mutated constructs, κB2, κB3 and κB1-4 were used for transfection of CD4$^+$CD25$^-$ T cells. CD28 engagement failed to induce reporter activity, which supports the role of κB-binding sites in the trans activation of *FOXP3* promoter. RelA overexpression in CD28-stimulated or unstimulated T cells in the same experimental conditions induces a remarkable increase of promoter activity in the absence of stimulation (Fig. 3C, ten-fold, relative to the empty vector), which could be further increased by activation (14-fold).

CD28 signaling induces RelA recruitment on *FOXP3* promoter and histone acetylation

To verify whether signals from CD28 may promote the recruitment of RelA on FOXP3 chromatin region and histone acetylation [24], we performed chromatin immunoprecipitation (ChIP) assays with anti-RelA and anti-acetyl histone H4 in CD4$^+$ CD25$^-$ T cells cultured with Dap3 or Dap3/B7 for different times. While a constitutive presence of RelA was not modified by activation in the region between −509 and −244, an increase of RelA could be shown on the region between −971 and −595 at 4 h after CD28 activation, was maximal after 8 h and decreased after 24 h (Fig. 4A). A slight binding of RelA was also obtained after 8 h on the region between −20 and +246. On the contrary, there was no apparent binding of RelA to region between −1517 and −1383. A significant association of RNA polymerase II with the promoter was evident after CD28 stimulation when RelA was

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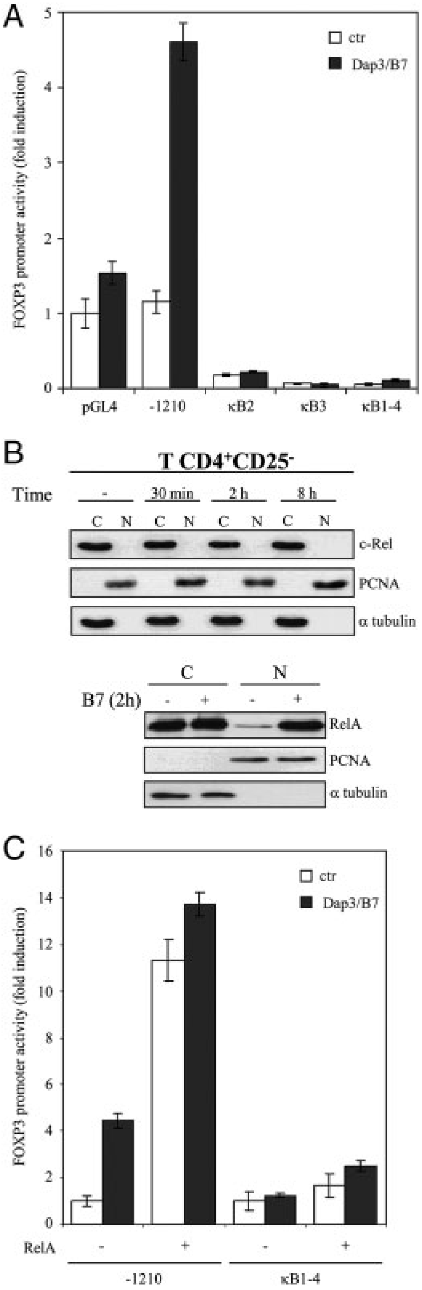

Figure 3. CD28 signaling trans activates *FOXP3* promoter expressed in CD4$^+$CD25$^-$ T cells by RelA. (A) CD4$^+$CD25$^-$ T cells, electroporated with pGL4 basic, FOXP3 −1210/+176 wt and with constructs containing κB-binding site mutations, were activated with Dap3 (ctr) or Dap3/B7 for 12 h. (B) CD4$^+$CD25$^-$T cells were stimulated with Dap3/B7 cells (B7) for the indicated times. Western blots for the presence of c-Rel (upper panel) and RelA/p65 (lower panel) were performed on cytoplasmic ("C") and nuclear ("N") extracts. Anti-PCNA and anti-α tubulin were used as loading control. Data shown are representative of three independent experiments. (C) CD4$^+$CD25$^-$ T cells were electroporated with FOXP3 −1210/+176 wt or κB1-4 mutant, co-transfected or not with RelA expression plasmid and activated. The results in (A) and (C) are calculated as luciferase units/protein concentration and represent the mean ± SD of one of three independent experiments performed in triplicate.

recruited on the promoter region and in the region downstream of TSS. To verify the activation of FOXP3 transcription after CD28 activation mRNA expression was measured in the cells used for the ChIP. CD28 signaling induced *FOXP3* mRNA expression as previously shown [19]. Moreover the binding of RelA to κB-binding sites at positions −873, −758 and +139 was verified by EMSA and by supershift analysis using an anti-RelA antibody (Supporting Information Fig. 6).

Figure 4B shows the results of ChIP assay with anti-acetyl histone. The histone H4 molecule was acetylated in the −971 −595 region of the *FOXP3* promoter after 8 h from CD28 stimulation, when the maximum recruitment of RelA on the same region occurred (Panel A). After 24 h, when a decrease of RelA binding occurs, the histone acetylation was not any more detectable. Moreover, histone H4 acetylation on region −20 +246 in resting CD4$^+$CD25$^-$ T cells has been also observed. Interestingly, we did not find histone H4 acetylation on region −510 −245, where Rel/NFAT-binding sites have been localized [12, 15].

We conclude that we have identified a region on human *FOXP3* promoter where RelA can be recruited following CD28 signaling and can contribute to the transcription of *FOXP3* gene by inducing histone H4 acetylation and subsequent loading of RNA polymerase II. This region is distinct from that where AP-1 and Rel-NFAT-binding sites have been characterized [15].

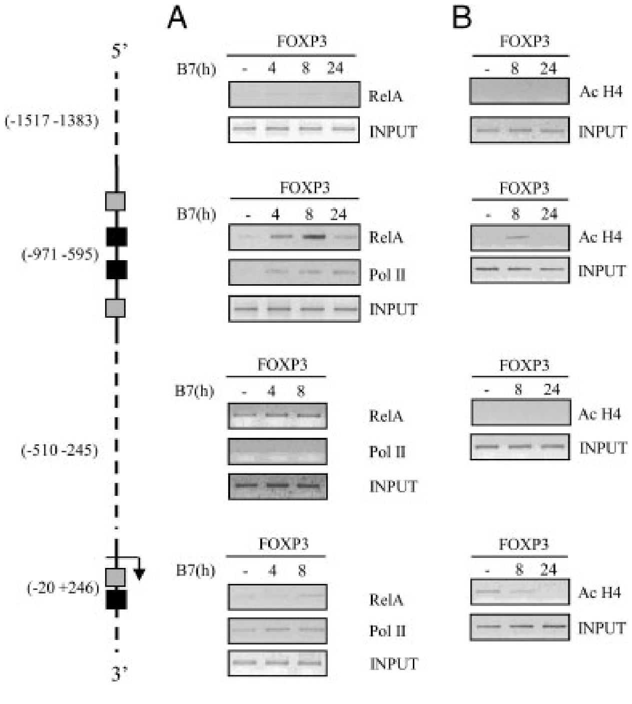

Figure 4. CD28 signaling in CD4$^+$CD25$^-$ T cells induces the recruitment of RelA on the natural *FOXP3* promoter and histone acetylation. CD4$^+$CD25$^-$T cells were stimulated with adherent Dap3/B7 cells for different times. ChIP assay was performed by using anti-RelA or anti-Pol II (A) or anti- acetyl-histone H4 Abs (B). Immunoprecipitated DNA was analyzed by PCR with different promoter-specific primers. The INPUT represents PCR amplification of the total sample, which was not subjected to any precipitation. Results shown are representative of three independent experiments. Scheme of the 5′ UTR region of *FOXP3* gene with the positions of putative NF-κB sites is shown.

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NFAT and NF-κB inhibition affects the trans activation of *FOXP3* promoter expressed in CD4$^+$CD25$^-$ T cells

To investigate the influence of NFAT and NF-κB factors on the activation of *FOXP3* promoter, we analyze the transcriptional activation of *FOXP3* promoter construct expressed in CD4$^+$CD25$^-$ T cells activated by PMA or Io or PMA/Io in the presence of CsA or NF-κB inhibitors. Indeed the signals provided by PMA have been described to be insensitive to CsA such as those mediated by CD28, unlike PMA/Io mediated signals are known to be sensitive to CsA such as those mediated by TCR. Figure 5A shows that, differently from Io that does not modify promoter activity, PMA induces a remarkable increase of promoter activity (70-fold$\pm$10 compared with the corresponding unstimulated cells) that was not significantly modified in the presence of CsA. However, promoter activity induced by PMA/Io was more than double of that induced by PMA (170-fold$\pm$27). Moreover, CsA induced a relevant inhibition of luciferase activity (65%) although the residual promoter activity activation was similar to that obtained with PMA and CsA. To confirm these results under conditions of physiological T-cell stimulation, CD28 or CD28 plus CD3 were stimulated by specific mAbs. Supporting Information Fig. 7 shows that the CsA inhibited the promoter activity induced by anti-CD3 and anti-CD28 (42%), but failed to modify its activity induced by anti-CD28, confirming that CsA resistant signals concur in the activation of *FOXP3* promoter. To investigate the effect of inhibition of NF-κB activation on *FOXP3* promoter, we analyzed the effect of ectopic DN IκB and of BAY11-7082 and *N-p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) inhibitors on −1210/+176 construct luciferase activity induced by PMA/Io in CD4$^+$CD25$^-$ T cells. Despite the activation of calcineurin-dependent transcription factors that favored −1210/+176 construct trans activation (Fig. 5A), NF-κB inhibition completely blocked *FOXP3* −1210/+176 luciferase activity (Fig. 5B and C). To verify whether the RelA-binding sites located in the region −971 to −595 concur to *FOXP3* promoter activation, the κB1-4-mutated constructs were transfected in CD4$^+$CD25$^-$ T cells (Fig. 5D) and luciferase activity measured at different times of stimulation by PMA/Io. The data show a conspicuous inhibition of the promoter activity induced by PMA/Io. However, while the κB1-4-mutated constructs was unresponsive to PMA/Io after 4 h, a residual luciferase activity could be still measured after 12 and 24 h, supporting the need of other transcriptional-binding sites to increase *FOXP3* promoter activation [12, 15, 23].

FOXP3 expression induced by CD3 and CD28 signals requires NF-κB

Many recent data in mice [11–14] strongly support the view that NF-κB recruitment on *FOXP3* promoter was necessary to FOXP3 expression. To verify whether NF-κB factors could play a crucial role in the activation of the human native *FOXP3* promoter in CD4$^+$CD25$^-$ T cells stimulated by TCR and CD28, we measured the effect of NF-κB inhibitors on *FOXP3* mRNA and protein expression. The inhibition of the nuclear translocation of NF-κB factors by BAY11-7082 has been also verified (Supporting Information Fig. 8). In the presence of TPCK and BAY11-7082 TCR and CD28 signals failed to activate *FOXP3* mRNA synthesis (Fig. 6A) and the percentage of CD25$^+$FOXP3$^+$ T cells was dramatically inhibited (Fig. 6B). Cellular viability was not related with the inhibition *FOXP3* mRNA (data not shown). The evidence that NF-κB inhibitors abrogated *FOXP3* gene activation induced by TCR and CD28 signals is an important observation, consistent with the view that the recruitment of NF-κB on *FOXP3* promoter is required to FOXP3 transcription and translation [11–14].

To verify the influence of RelA in the activation of FOXP3 transcription, RelA was knocked down using small interfering RNA (siRNA) oligonucleotides against RelA. Cells were transfected with siRNA or with a scrambled siRNA as negative control and then cultured with anti-CD3 and anti-CD28 mAbs for 48 h. Dose response experiments were performed and the levels of RelA protein (Fig. 7A) and *FOXP3* mRNA (Fig. 7B) determined. The reduction of RelA due to siRNA treatment correlated with the decrease of FOXP3 expression. After 48 h, when the levels of RelA protein were undetectable, *FOXP3* mRNA was inhibited by 67% compared with scrambled siRNA-transfected cells. These data confirm the crucial role of RelA on FOXP3 expression and support the need of costimulatory signals in the activation of iTreg.

Discussion

The current study reveals that CD28 unique signals initiate the activation of *FOXP3* promoter via a mechanism that involves the activation and binding of RelA to specific, yet unknown, binding sites on *FOXP3* promoter, implying that CD28 costimulation may influence FOXP3 expression through epigenetic mechanisms independent of TCR-mediated activation of c-Rel and NFAT.

It has been demonstrated that a conserved fragment of 453 bp upstream of the FOXP3 TSS contains a minimal promoter necessary for induction of FOXP3 expression [15]. Therefore, this region has been considered critical for the transient expression of FOXP3 by activated T cells. However, we show that DNA region between −936 and −646 nucleotides of the human *FOXP3* promoter is characterized by at least three functional and not redundant κB sites for the NF-κB subunit RelA that contribute to *FOXP3* promoter activation as RelA homodimers. Indeed, disruption of each of these elements in the reporter constructs reduced the effect of RelA on the promoter, suggesting that the direct binding to multiple sites is necessary to mediate gene trans activation. However, these sites could not be required for c-Rel-mediated FOXP3 activation, since their deletion did not modify the effect of c-Rel on *FOXP3* promoter.

These data extend previous results to correlate the transcription and translation of FOXP3 in a small number of CD4$^+$CD25$^-$ T cells activated by CD28 signals independent of TCR-mediated stimulatory pathways with NF-κB factor activation [19]. In the context of the natural promoter, we have shown that CD28

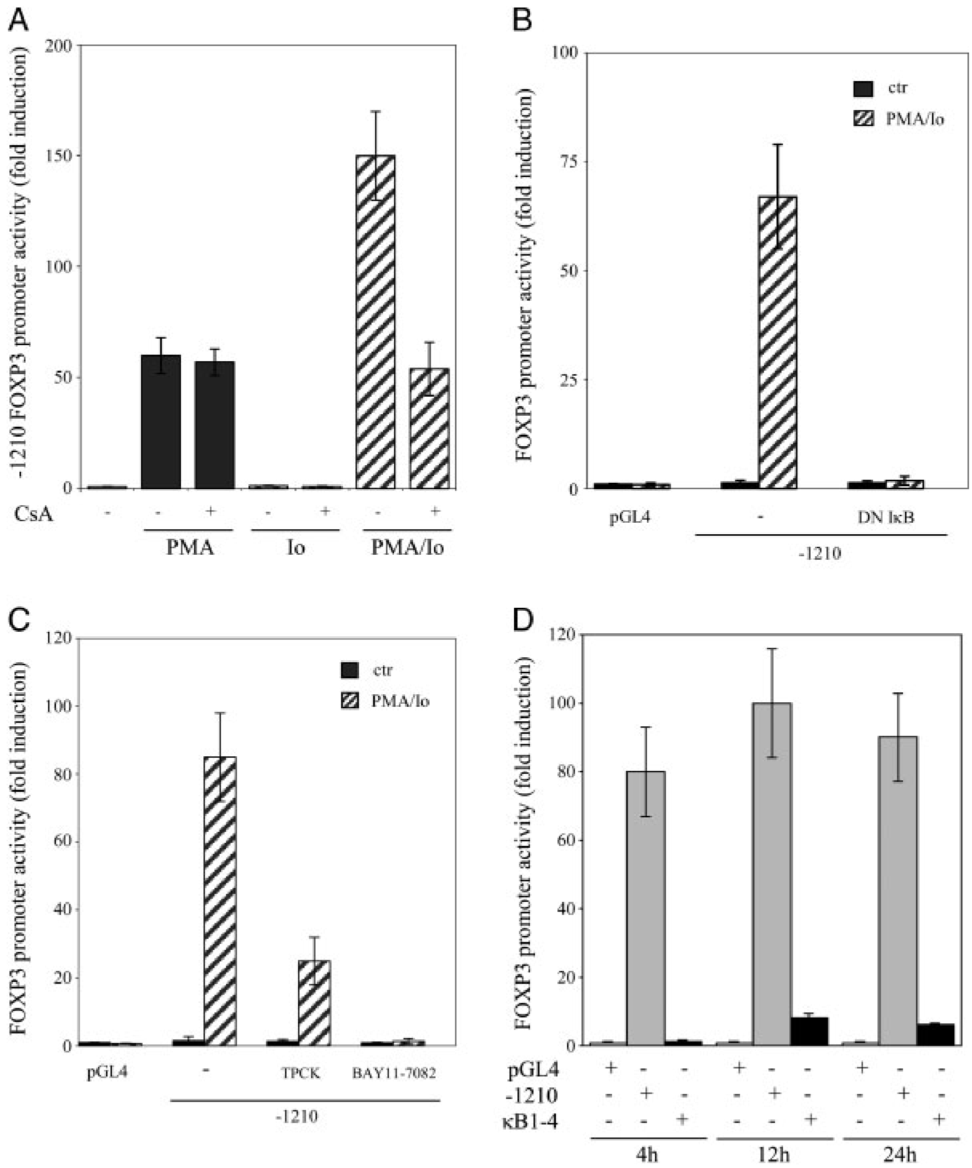

Figure 5. *FOXP3* promoter activity induced by PMA/Io is regulated by both CsA sensitive pathways and NF-κB. CD4$^+$CD25$^-$ T cells, electroporated with pGL4 basic or FOXP3 -1210/+176 promoter luciferase reporter vector, were treated with CsA (1 μg/mL) and activated with PMA or Io or PMA/Io (A), or co-transfected, where indicated, with construct containing dominant negative IκB (B), or treated or not with TPCK (25 μM) or BAY11-7082 (5 μM) (C) and activated or not (ctr) with PMA/Io. (D) CD4$^+$CD25$^-$ T cells, electroporated with pGL4 basic, FOXP3 −1210/+176 wt promoter luciferase vector or with κB1-4 mutant, were activated with PMA/Io for the indicated times. Luciferase activity was measured. Bars show the mean ± SD of three independent experiments.

unique signals are sufficient to mediate the translocation of RelA from the cytoplasm to the nucleus, its recruitment on κB-binding sites and the histone acetylation of the regions bound by RelA. CD28-mediated FOXP3 trans activation could occur in the absence of c-Rel, since CD28 pathways fail to activate c-Rel nuclear translocation [20]. Thus, RelA activated by CD28 plays an important role in the trans activation of *FOXP3* gene and its expression. Conversely, many reports agree that c-Rel is the critical NF-κB transcription factor required to generate normal number of thymic and peripheral Tregs [11–14]. It has been postulated that the recruitment of c-Rel on *FOXP3* promoter could initiate FOXP3 transcription, while the contribution of RelA is not clear [11, 12]. The reasons for this discrepancy between our and others reports could be various, including the differences in stimulation conditions or cell types; however, we did not find a high conservation between mice and humans in the *FOXP3* DNA region where novel RelA sites have been identified suggesting that murine *FOXP3* promoter could be less dependent on RelA. Moreover, another explanation could be that the development of Tregs in the thymus and maintenance of Treg phenotype have distinct molecular requirements, and c-Rel is important in regulating thymic Treg differentiation [14].

Interestingly both c-Rel and RelA trans activated human *FOXP3* promoter. Moreover, the activation of TCR and CD28

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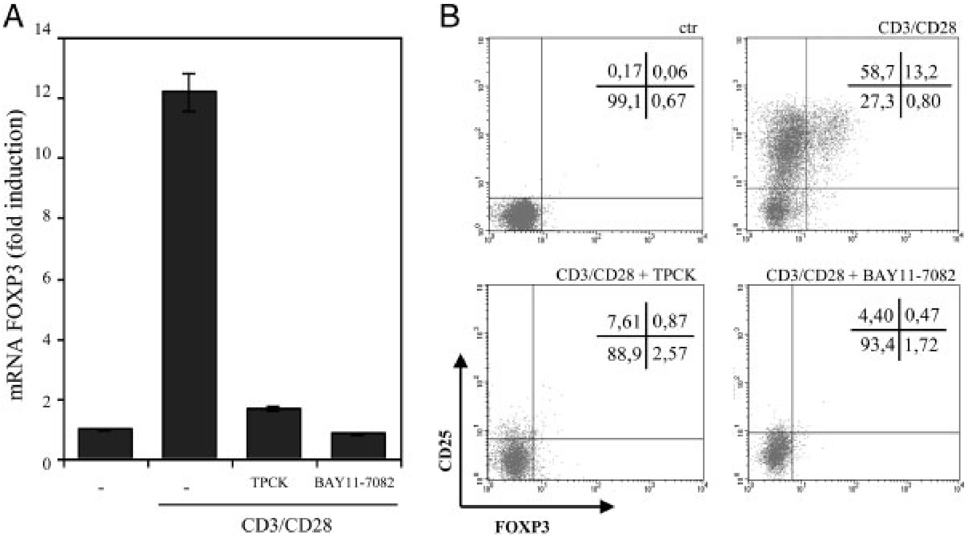

Figure 6. CD3 and CD28 signals fail to activate FOXP3 expression in the presence of NF-κB inhibitors. CD4$^+$CD25$^-$ T cells were pre-treated with NF-κB inhibitors and activated with anti-CD3 plus anti-CD28 mAbs. *FOXP3* mRNA level was measured after 24 h by real-time PCR and bars show the mean ± SD of one representative experiment performed in triplicate out of three (A). FOXP3 and CD25 expression was measured after 48 h by FACS analysis. Viable lymphocytes were identified by their forward scatter (FSC) versus side scatter (SSC) profile. CD4$^+$ lymphocytes were analyzed by their single positive, double positive and double negative profiles according to expression of CD25 and FOXP3. Corresponding isotype controls were used to set up the quadrant positions. The dot plots are representative of three independent experiments (B).

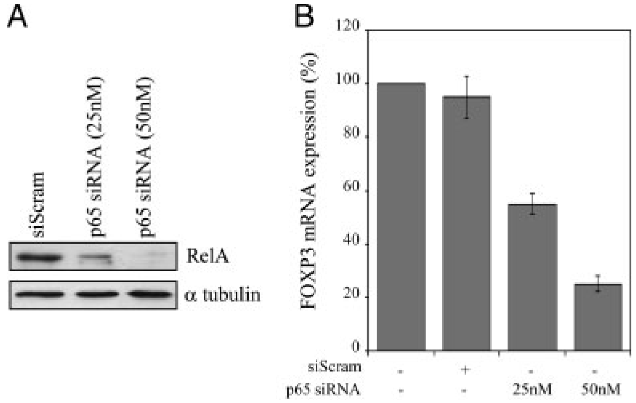

Figure 7. siRNA-mediated knockdown of RelA/p65 decreases FOXP3 expression. CD4$^+$CD25$^-$ T cells were transfected with p65 siRNA or with a scrambled siRNA as negative control. Twelve hours posttransfection, the cells were stimulated with anti-CD3 and anti-CD28 mAbs. Immunoblots were used to measure p65 knockdown (A). mRNA levels of FOXP3 were determined by RT-PCR after 48 h. Levels in untreated cells were set to 100%. Bars represent mean and SD of at least three independent experiments (B).

signaling pathways increased the trans activation of *FOXP3* promoter expressed in human CD4$^+$CD25$^-$ T cells. This implies that TCR and CD28 may induce the sequential and independent activation of both RelA and c-Rel by using different pathways that synergize in the activation of FOXP3. Indeed, although CD28 signaling is thought to be concomitant with TCR engagement, it occurs that TCR and CD28 are independent signaling units and biophysical and biochemical comparisons show differences in their signaling scope [25]. In T cells engagement of CD28 induces IκBα-degrading signals, that mediate only RelA activation [20], while nuclear c-Rel expression requires new gene transcription and translation by a pathway that is suppressed by CsA [26]. This mechanism results in a slower c-Rel induction and, consequently, slower c-Rel-dependent gene transcription compared to that mediated by RelA. Therefore, CD28 unique signals could mediate a fast engagement of κB sites by RelA for initiating FOXP3 transcription, but maximal transcription will occur only upon c-Rel recruitment.

FOXP3 promoter shows a stronger association with acetylated histones in Treg than in conventional T cells [15, 17], suggesting that in order to induce the activation of the *FOXP3* gene, the histones that cover *FOXP3* DNA have to be acetylated. Therefore, another function of the CD28 mediated recruitment of RelA on the specific binding sites could be the induction of epigenetic modification of the *FOXP3* gene at the level of DNA region distinct from the region where several important transcription factor-binding sites have been identified. The histone acetylation of this region may be compatible with at least two scenarios. First, it is required to mediate the recruitment of Pol II and to initiate FOXP3 transcription independently from TCR. Second, since TCR signaling in the absence of CD28 costimulation did not result in any epigenetic change [24], RelA could favor the promoter occupancy by other transcription factors. The evidence that siRNA-mediated knockdown of RelA inhibited trans activation of *FOXP3* promoter in CD4$^+$CD25$^-$ T cells full activated by TCR and CD28 sustains this scenario. Moreover, in the absence of the binding of RelA to *FOXP3* promoter, NFAT failed to mediate trans activating function, suggesting that RelA and not NFAT is primarily required to trans activate *FOXP3* gene, and that the occupancy of κB sites favors the recruitment of NFAT on the specific binding sites. Recent data

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describing the transcriptional activation at gene promoters by NF-κB support this possibility [27].

In mice, many data indicate that the TCR-NF-κB axis controls the development of natural Treg [9, 10]. In particular, genetic disruption of caspase recruitment domain-containing membrane-associated guanylate kinase protein-1 (CARMA1) resulted in normal development of conventional $CD4^+$ and $CD8^+$ cells but complete absence of natural Treg [9]. Therefore, our evidence that inhibitors of NF-κB processing abolished the trans activation of *FOXP3* promoter and its expression despite the activation of TCR and CD28 pathways represents a further demonstration of the essential requirement for the expression of FOXP3 resides in NF-κB proteins. However, it has also been proposed that the activation of NF-κB by TCR is not required for FOXP3 expression [9]. Transient TCR stimulation and inhibition of PI3K-AKT-mammalian target of rapamycin (mTOR) signaling axis can enhance in vitro FOXP3 expression [28]. Since CD28 is a principal activator of NF-κB pathways and the activation of the NF-κB signaling pathway via CARMA1-Bcl10 may be markedly dependent on CD28 stimulation rather than TCR stimulation [29], CD28 rather than TCR may control FOXP3 expression.

In conclusion, the current study reveals a new mechanism of *FOXP3* gene regulation, and supports a scenario where CD28-mediated recruitment of RelA on *FOXP3* gene is necessary for the induction of FOXP3 transcription in $CD4^+CD25^-$T cells, while supplementary signals such as those mediated by TCR are required to allow sustained and maximal FOXP3 synthesis. On the basis of our data, it will be possible to explore receptors and signaling partners that may give access to specific interventions aiming at Treg.

Materials and methods

Cell lines, Abs and reagents

Murine L cells (Dap3) and murine L cells expressing human natural ligand B7.1 (Dap3/B7) were grown in DMEM (Gibco-BRL, Grand Island, NY) containing 10% FBS supplemented with 50 μg/mL hygromycin B (Sigma Aldrich, St. Louis, MO). HEK 293 cells were maintained in DMEM supplemented with 10% FBS. Inhibitors used were TPCK (Calbiochem), BAY11-7082 (Calbiochem) and CsA (Sigma Aldrich). Anti-CD3 (UCHT1) and anti-CD28.2 mAbs were purchased from BD Biosciences (San Jose, CA), goat anti-mouse (Poly 4053) Ab was purchased from Biolegend (San Diego, CA). Anti-NFκB/p65 (C-20), anti-c-Rel (sc-70), anti-NFATp (4G6-G5) and anti-α tubulin (B-7) Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-PCNA Ab was purchased from Cell Signaling (Beverly, MA). FITC-conjugated anti-human CD4 (MEM-241) Ab was obtained from Immunological Science. FITC-conjugated anti-human CD25 (M-A251), PE-conjugated anti-human *FOXP3* (259D/C7) and anti-mouse IgG1 isotype control mAbs were purchased from BD Pharmigen (San Diego, CA).

$CD4^+CD25^-$ T cells isolation and activation

PBMCs were isolated from buffy coats of healthy donors by centrifugation over Lympholyte-H (Cederlane, Hornby, Canada) gradients. $CD4^+CD25^-$ T cells were purified by human Treg isolation kit (Miltenyi Biotec, Auburn, CA) and cultured as previously described [19]. The purity of $CD4^+CD25^-$ T-cell population was confirmed to be >95% by flow cytometry.

$CD4^+CD25^-$ T cells were activated either with Dap3 (used as control) or Dap3/B7, as previously described [19]. Alternatively, $CD4^+CD25^-$ T cells were activated with 2 μg/mL of anti-CD28 mAb cross-linked with 10 μg/mL of goat anti-mouse Ab in plates uncoated or coated with anti-CD3 (5 μg/mL) mAbs or with PMA (50 ng/mL) and ionomycin (2 μg/mL).

Plasmids, cell transfection and luciferase assays

The human *FOXP3* promoter constructs were cloned into pGL4 vector (Promega) to generate the pGL4 FOXP3 luciferase reporter vectors as previously described [15]. NFATp expression plasmid was kindly provided by Cippitelli M. (Regina Elena Cancer Institute, Rome, Italy). The κB site binding-defective mutant p65/RelA 23Y>A; 26E>D (RelA YA ED) [22] and HA-tagged cRel were kindly provided by Natoli G. (Department of Experimental Oncology, European Institute of Oncology, Milan, Italy). The dominant negative IκB was from Upstate Biotechnology (Lake Placid, NY).

The κB-binding site mutants were derived from FOXP3 −1210/+176 promoter luciferase construct by substituting five nucleotides within the consensus-binding sites and deleting two NF-κB-binding sites, by PCR. Primers used to generate the individual constructs are listed in Supporting Information Table 1. The entire sequences of the mutants were verified by DNA sequencing.

HEK 293 cells were transfected by electroporation using 5 μg of total DNA in 450 μL of DMEM supplemented with 20% FBS. Electroporation was performed in 0.45-cm electroporation cuvettes (Gene pulser; BioRad, Hercules, CA) at 960 μF and 250 V. After 24 h, luciferase activity was measured according to the manufacturer's instruction (Promega). $CD4^+CD25^-$ T cells, resuspended in 100 μL of Nucleofector solution (Amaxa Biosystems), were electroporated with *FOXP3* promoter luciferase reporter vectors using the V-024 program of the Nucleofector. For the co-transfection, 1.5 μg of *FOXP3* promoter luciferase reporter vector and 3 μg of p65/RelA expression plasmid were used. After stimulation as indicated in the figures, the luciferase activity in cell lysates was measured. Luciferase activity determined in triplicates was expressed as fold induction over the basal activity of cells transfected with empty vectors and/or with *FOXP3* promoter luciferase construct after normalization to protein concentration. All co-transfections were performed in the presence of 0.5 μg of pEGFP expression plasmid (Clontech). On average, ~35% of the cells expressed the transfected plasmids, as determined by fluorescence-activated cell sorter (FACS) analysis of green fluorescent protein (GFP)-cotransfected cells.

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Quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) from 3×10^6 purified T cells and was reverse-transcribed into cDNA by using Moloney murine leukemia virus reverse transcriptase (Invitrogen). TaqMan Universal PCR Master Mix and the FOXP3 primer/probe set (part No. Hs00203958_m1) and the GAPDH primer/probe set (part No. Hs99999905_m1) were purchased from Applied Biosystems and the relative quantification was performed using the comparative C_T method.

Immunoblotting

$CD4^+$ T cells were stimulated for the indicated times and both cytoplasmic and nuclear extracts were prepared as previously described [20]. Proteins were resolved by 10% SDS-PAGE and blotted onto nitrocellulose membranes. Blots were incubated with the indicated primary Abs and, after incubation with horseradish peroxidase-labeled goat anti-rabbit or horseradish peroxidase-labeled goat anti-mouse (Amersham), developed with the enhanced chemiluminescence detection system (Amersham).

ChIP assay

ChIP assays were performed as previously described [19]. For precipitation Abs against NFκB/p65 (C-20), Pol II (N-20) (Santa Cruz, CA) or acetyl-histone H4 (Upstate Biotechnology) were used. Primers, addressing the FOXP3 promoter regions −1517 to −1383, −971 to −595, −510 to −245 and −20 to +246, were, respectively, as follows: 5′-AAGTCTTCACCATGGCCTGTCAG-3′ and 5′-GCTTGTGAGGCTTTTAGGTTGTCC-3′; 5′-GCATCAGGCCATGATGTTCCTGAA-3′ and 5′-ATGGTCCCCATTGCTTGAACTACC-3′; 5′-TTCCCATCCACACATAGAGC-3′ and 5′-TGCCCTTTACGAGTCATCTG-3′; 5′-GATACGTGACAGTTTCCCAC-3′ and 5′-CCCTGCCCATGCATTAAGTA-3′.

Immunofluorescent staining and flow cytometry analysis

Cells (2×10^5) 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 mAb or isotype control according to the manufacturer's instructions and subjected to FACS analysis [19].

RNA interference

Transfections were performed using predesigned siRNAs against p65(Ambion;S11914 and S11915) or a non-specific negative control (Ambion;4390843). A total of 10×10^6 $CD4^+CD25^-$ T cells, were transfected in 100 μL of Nucleofector solution, using the V-024 program. siRNA oligos were transfected at a concentration of 25 and 50 nM. Two different siRNA oligos were tested (data not shown) and oligo siRNA S11915 was used in subsequent experiments. Twelve hours posttransfection with siRNA, the medium was replaced with fresh, and cells were stimulated with anti-CD3 plus anti-CD28 mAbs.

Statistical analysis

Data were analyzed by the Student's *t*-test. A value of $p<0.05$ was considered significant statistically.

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Abbreviations: **CsA:** cyclosporine A · **FOXP3:** forkhead box P3 · **iTreg:** induced Treg · **TPCK:** *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone · **TSS:** transcription start site

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