

Regulation of the *foxp3* Gene by the Th1 Cytokines: The Role of IL-27-Induced STAT1¹

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Impaired functional activity of T regulatory cells has been reported in allergic patients and results in an increased susceptibility to autoimmune diseases. The master regulator of T regulatory cell differentiation, the transcription factor FOXP3, is required for both their development and function. Despite its key role, relatively little is known about the molecular mechanisms regulating *foxp3* gene expression. In the present study, the effect of Th1 cytokines on human T regulatory cell differentiation was analyzed at epigenetic and gene expression levels and reveals a mechanism by which the STAT1-activating cytokines IL-27 and IFN- γ amplify TGF- β -induced FOXP3 expression. This study shows STAT1 binding elements within the proximal part of the human FOXP3 promoter, which we previously hypothesized to function as a key regulatory unit. Direct binding of STAT1 to the FOXP3 promoter following IL-27 stimulation increases its transactivation process and induces permissive histone modifications in this key region of the FOXP3 promoter, suggesting that FOXP3 expression is promoted by IL-27 by two mechanisms. Our data demonstrate a molecular mechanism regulating FOXP3 expression, which is of considerable interest for the development of new drug targets aiming to support anti-inflammatory mechanisms of the immune system. *The Journal of Immunology*, 2009, 182: 1041–1049.

Differentiation of effector T cell subsets is an important process preceding specific inflammatory immune responses triggered by invading pathogens. The differentiation of uncommitted CD4⁺ T cells toward different Th cell subsets is determined by lineage-specific transcription factors, such as the Th1-specific T-bet for Th1 cells (1), the GATA-binding protein 3 (GATA-3)³ for Th2 cells (2), and the retinoic orphan receptor C2 for Th17 cells (3, 4). These transcription factors coordinate the production of a specific cytokine profile (e.g., Th1: IFN- γ and TNF- α ; Th2: IL-4, IL-5, IL-9, and IL-13; Th17: IL-17, IL-22, IL-26, and IL-6) and define the functionality of the subsets (5). Although Th1, Th2, and Th17 cells are specialized in immunity during viral, parasitic, and other infections, T regulatory (T_{reg}) cells are dedicated in the control of immune responses and mediate

tolerance against harmless non-self or self-Ags (6). The differentiation and function of T_{reg} cells require the transcription factor forkhead box p3 (FOXP3) (7, 8) and inducible T_{reg} (iT_{reg}) cells can be generated in the periphery from CD4⁺CD25[−] naive T cells (9–13). Despite its critical function, relatively little is known about the molecular mechanisms regulating *foxp3* gene induction. Various studies investigated the physiological inducers of FOXP3 expression in T cells, and TGF- β was shown to be essential for the induction of FOXP3⁺ iT_{reg} cells (11). However, it remained unclear whether its effect originates from direct interactions with the *foxp3* gene. We recently characterized the FOXP3 promoter and identified NFAT and AP-1 binding sites that act as TCR-responsive units of the *foxp3* gene (14, 15). In agreement with these findings, it was shown that Smad3 and NFAT cooperatively induce FOXP3 and regulate the chromatin availability of its enhancer region (16). Alternatively, TGF- β may act via TGF-inducible early gene 1 product and the E3 ubiquitin ligase Itch to participate in an ubiquitin-dependant pathway regulation of the *foxp3* gene (17). In addition to TCR-induced NFAT and AP-1, the Sp-1- and IL-2-induced STAT5 bind to the promoter and positively regulate its expression (18–20). Several GATA-3 binding sites within the FOXP3 promoter negatively regulate FOXP3 expression, suggesting that IL-4-induced Th2 cell differentiation overrule T_{reg} cell differentiation (14). Consequently, it could be hypothesized that Th1 responses also restrict FOXP3 expression and T_{reg} cell differentiation. However, this hypothesis was not thoroughly analyzed, partly due to the complexity of Th1 differentiation involving multiple cytokines such as IL-12, IL-27, and IFN- γ . It has been shown that IFN- γ is required for FOXP3 expression and for conversion of CD4⁺CD25[−] T cells into T_{reg} cells in an experimental autoimmune encephalomyelitis model (21). Initially, IL-27 was described to be implicated in early events controlling Th1 cell differentiation by inducing IL-12R β 2 expression in a STAT1/T-bet-dependent manner (1, 22). This heterodimeric cytokine is composed of p28 and Epstein-Barr-induced gene 3 (EBI3) subunits, where p28 is related to IL-12p35 with a classical cytokine structure

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³ Abbreviations used in this paper: GATA-3, GATA-binding protein 3; ChIP, chromatin immune precipitation; FOXP3, human forkhead box p3; Foxp3, mice forkhead box p3; T_{reg}, T regulatory; iT_{reg}, inducible T_{reg}; TSS, transcription start site.

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and EBI3 to IL-12p40 with structural resemblance to the soluble IL-6R chain (22). Recently, IL-27 has emerged as an important immunoregulatory cytokine, which antagonizes the development of Th17 cell responses during experimental autoimmune encephalomyelitis and limits the IL-17-driven inflammation in CNS as well as in models of uveitis and scleritis (23–25). Even though IL-27 has wide-reaching roles in immune responses in a STAT1-dependent manner (24, 26–28), the exact mechanisms by which IL-27 exerts regulatory functions are so far unknown.

Motivated by the importance of FOXP3 in the control of immune responses, we investigated the influence of Th1 cytokines on FOXP3 expression. In this study, we demonstrate that IL-27 has the capability to act on naive CD4⁺ T cells during their differentiation into effector cells. When the IL-27 stimulation is coupled with TGF- β stimulation, cells undergo iT_{reg} differentiation with reduced IFN- γ production and potently suppressive capacity. Part of the mechanism leading to this differentiation is mediated via the induction of FOXP3. Phosphorylation of STAT1 on Tyr⁷⁰¹ following IL-27 stimulation enables its binding to the sites at positions –99 and –7 bp of the FOXP3 promoter, which increases the expression of the *foxp3* gene via epigenetic regulation of the promoter.

Materials and Methods

T cell isolation

PBMC were isolated from buffy coats of healthy donors by Ficoll (Biochrom) density gradient centrifugation. CD4⁺T cells were purified with anti-CD4-Dynal magnetic beads and Detach-a-Bead Abs (Dynal) according to the manufacturer's instructions. CD4⁺CD45RA⁺ cells were purified by MACS (Miltenyi Biotec). CD4⁺CD45RA⁺ T cell purity was consistently $\geq 90\%$ as determined by FACS analysis.

In vitro T cell differentiation

All T cells were maintained in AIM-V serum-free medium (Life Technologies) with 30 ng/ml IL-2 (PeproTech) and polyclonally stimulated with soluble anti-CD3 (2.5 μ g/ml; clone Okt3; IgG1) and anti-CD28 (2.5 μ g/ml; clone B7G5). Naive T cells were driven under neutral condition: 5 μ g/ml anti-IL-4, 5 μ g/ml anti-IL-12, and 1 μ g/ml anti-IFN- γ ; under Th1 condition: 5 μ g/ml anti-IL-4 and 25 ng/ml IL-12; under Th2 condition: 5 μ g/ml anti-IL-12, 1 μ g/ml anti-IFN- γ , and 25 ng/ml IL-4; or under iT_{reg} condition: 5 μ g/ml anti-IL-4, 5 μ g/ml anti-IL-12, 1 μ g/ml anti-IFN- γ , and 5 ng/ml TGF- β with or without 160 ng/ml IL-27, 25 ng/ml IL-12, or 1000/ml IU IFN- γ (R&D Systems). Proliferating cells were expanded in medium containing IL-2 (30 ng/ml).

RNA isolation and cDNA synthesis

RNA was isolated using a RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Reverse transcription was performed with Fermentas reverse transcription reagents with random hexamers according to the manufacturer's protocol.

Quantitative real-time PCR

The PCR primers and probes detecting FOXP3 were designed based on the sequences reported in GenBank with the Primer Express software version 1.2 (Applied Biosystems), as listed in supplementary Fig. 5.⁴ The prepared cDNAs were amplified using SYBR-PCR Mastermix (Bio-Rad) according to the recommendations of the manufacturer in an Applied Biosystems PRISM 7000 Sequence Detection System. Relative quantification and calculation of the range of confidence were performed using the comparative $\Delta\Delta$ CT method, as previously described (14, 15). All amplifications were conducted in triplicates.

Flow cytometry

Cells were stained with the mAb CD25 (Beckman Coulter) prior to the FOXP3 intracellular staining which was performed according to the manufacturer's protocol using an Alexa Fluor 488 anti-human FOXP3 Flow kit clone (295D; Biolegend).

Intracellular cytokine labeling was performed with R-PE-conjugated mouse anti-human IFN- γ mAb (clone B27; BD Biosciences). Cells were stimulated with PMA (25 ng/ml), ionomycin (1 mg/ml), and brefeldin A (10 mg/ml; Sigma-Aldrich) 5 h before staining. Cells were fixed and permeabilized using a BD Biosciences Cytofix/Cytoperm kit according to the manufacturer's instructions. Matched isotype controls were used at the same protein concentration as the respective Abs.

Cell acquisition by flow cytometry was done on a four-color FACS EPICS XL-MCL (Beckman Coulter) using the software Expo 32 version for data acquisition and evaluation.

Cytokine quantification

Supernatants of in vitro-differentiated cells that were restimulated at day 12 with anti-CD3 (2.5 μ g/ml) and anti-CD28 (2.5 μ g/ml) were analyzed for cytokines using a Bio-Plex System according to the manufacturer's protocol (Bio-Rad).

Suppression assay

CD4⁺CD45RA⁺ T cells were stimulated under iT_{reg} conditions during 7 days, washed, and rested in IL-2-containing medium during 3 days. At that point, autologous PBMC and CD4⁺T cells were isolated. CD4⁺ responder T cells were washed twice with PBS and labeled in PBS/2 μ M CFSE (Molecular Probes and Invitrogen) for 3 min at room temperature. Cells were washed twice with complete RPMI 1640 (Life Technologies). A fixed number of 1×10^5 CD4⁺ T cells (iT_{reg} plus responder cells) were cocultured with 1×10^5 irradiated PBMC in 96 round-bottom plates with anti-CD3 (2.5 μ g/ml) during 5 days. The number of iT_{reg} cells corresponds to the total number of naive CD4⁺ T cells that were cultured under T_{reg}-driven condition with or without IL-27, IL-12, or IFN- γ for 10 days. The proliferation of CD4⁺ responder T cells was analyzed by flow cytometry.

Bioinformatics

Transcription factor binding sites were identified using TESS software (www.cbil.upenn.edu/cgi-bin/tess/tess33), which uses matrices of the Transfac database.

Western blotting

Nuclear extraction was performed as follows. All buffers contained EDTA-free complete protease inhibitor (Roche Diagnostics). Cells were resuspended in 400 μ l of buffer A (10 mM KCl, 1 mM DTT, 10 mM HEPES (pH 7.9), and 1 mM EDTA). After 15 min of incubation, 25 μ l of 10% Nonidet P-40 (Sigma-Aldrich) was added and the tube was vortexed vigorously for 30 s followed by a centrifugation step (1 min, maximum speed, 4°C). The translucent nuclear pellet was washed once with 1 ml of buffer A, resuspended in 50 μ l of buffer B (400 mM NaCl, 1 mM DTT, 20 mM HEPES (pH 7.9), and 1 mM EDTA), and incubated for 15 min at 4°C on a roller. Nuclear debris and genomic DNA were removed by centrifugation (5 min, maximum speed, 4°C). The supernatant was diluted 1/3 with buffer D (20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT, and 0.1% Nonidet P-40). Total protein concentration was determined by a colorimetric protein assay (Bio-Rad). Samples were loaded next to a protein-mass ladder (Fermentas) on a NuPAGE 4–12% bis-Tris gel (Invitrogen Life Technologies). The proteins were electroblotted onto a polyvinylidene difluoride membrane (Amersham Biosciences). Unspecific binding was blocked with TBS-Tween 20 with 3% milk. After blocking, the membranes were incubated with the primary Ab in blocking buffer overnight at 4°C. The blots were developed using the proper secondary HRP-labeled Ab and visualized with an LAS 1000 camera (Fuji). To confirm sample loading and transfer, membranes were incubated in stripping buffer and reblocked for 1 h and then reprobed using anti-GAPDH (6C5; Ambion). STAT1 P-Tyr⁷⁰¹, STAT3 P-Tyr⁷⁰⁵, STAT4 P-Tyr⁶⁹³, STAT5 P-Tyr⁶⁹⁴ rabbit Ab, and anti-rabbit HR- labeled Ab (Cell Signaling).

Amplification of FOXP3 promoter fragments

FOXP3 promoter fragments were amplified by conventional PCR using the biotinylated reverse primer 5'-bio-ACCTTACTGGCTGGAATCAGC-3' situated 177 bp downstream of the transcription start site (TSS). Multiple forward primers were designed to generate FOXP3 promoter fragments of different lengths (supplementary Fig. 5). Reactions were conducted in 75 mM Tris-HCl (pH 8.8), 20 mM (NH₄)₂SO₄, 2 mM MgCl₂, 0.2 mM dNTPs, 0.2 μ M of each primer, 6 μ g/ml template DNA (FOXP3 promoter in pGL3), and 1.25 U of recombinant TaqDNA polymerase (Fermentas). The same PCR conditions were used for the amplification of all of the products: initial denaturation step (2 min, 94°C), 42 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 60 s, final elongation step (7 min, 72°C). PCR products were purified by ethanol precipitation. Therefore, 1/10 volume of 3 M sodium

⁴ The online version of this article contains supplemental material.

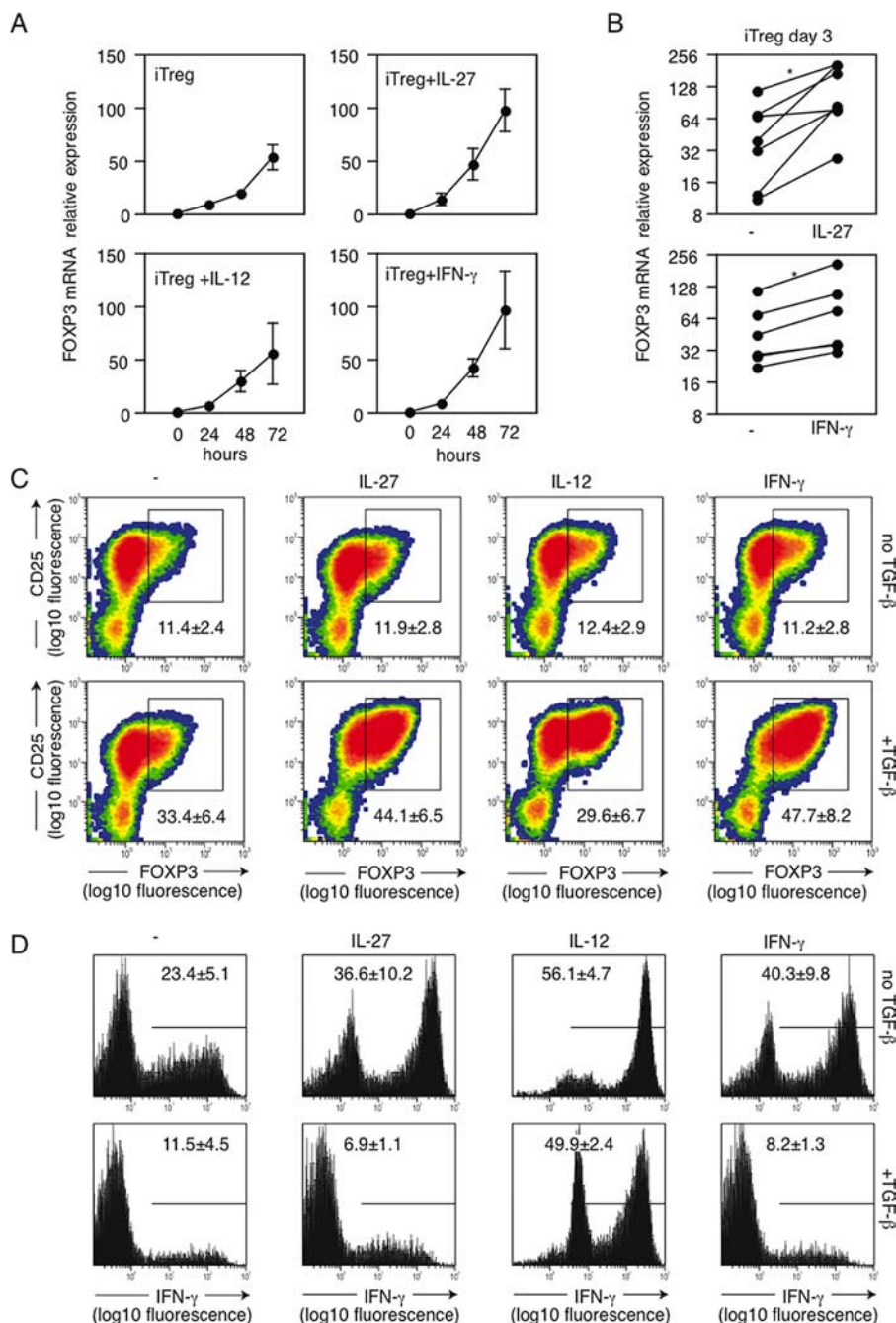


FIGURE 1. IL-27 and IFN- γ increase TGF- β -induced FOXP3 expression without inducing Th1 differentiation. Human CD4⁺CD45RA⁺ T cells were activated with soluble anti-CD3/CD28 and differentiated in iTreg-driven conditions with or without IL-27, IL-12, or IFN- γ as indicated. Cells were harvested and FOXP3 expression was analyzed by real-time PCR after 24–72 h (A) or 3 days (B) and γ FACS analysis (C) along with CD25 expression after 5 days. D, After 12 days, intracellular IFN- γ expression was analyzed by FACS following PMA/ionomycin stimulation. FACS data are representative of five independent experiments. Symbols represent the mean $\pm$ SEM of six to eight independent experiments (A) or different donors (B). *, $p < 0.05$.

acetate and three volumes of 100% ethanol was added to the PCR products, mixed, and frozen for 1 h at -80°C . Unfreezing was followed by a centrifugation step (30 min, maximum speed, 4°C). The supernatant was removed and residual ethanol was allowed to evaporate. The pellet was eluted in blocking buffer (PBS, 0.1% BSA, and 0.05% Tween 20). Quantification of the FOXP3 promoter probe was done with a MassRuler DNA Ladder Mix (Fermentas) on a 1% agarose gel using AIDA image analyzer software (Raytest).

FOXP3 promoter ELISA

Nuclear extraction was performed as follows. The cells were pelleted and resuspended in buffer C (20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT, protease inhibitors (Roche Diagnostics), and 0.1% Nonidet P-40) and lysed on ice for 15 min. Insoluble material was removed by centrifugation. The supernatant was diluted 1/3 with buffer D (as buffer C, but without NaCl). Three hundred eighty-four-well plates precoated with streptavidin (Pierce) were washed three times with washing buffer (PBS and 0.05% Tween 20). The biotinylated FOXP3 promoter oligonucleotides or the biotinylated STAT consensus sequence oli-

gonucleotides probes (supplementary Fig. 5) were added (1 pmol/well; 50 fmol/ μl) and incubated for 1 h at room temperature. After three washings with washing buffer, nuclear extract was added (concentration $>0.2 \mu\text{g}/\mu\text{l}$) and incubated overnight at 4°C . The lysates were incubated with 10 μg of poly(deoxyinosinic-deoxycytidylic acid) (Sigma-Aldrich). The plate was washed three times with buffer C/D and primary Ab (rabbit anti STAT1, 1/200 in buffer C/D; Cell Signaling) was added and incubated at 4°C for 2 h. After three washing steps with buffer C/D, secondary Ab (anti-rabbit IgG-HRP, 1/3000 in buffer C/D; Cell Signaling) was added and the plate was incubated for 1 h at 4°C . The wells were washed four times with buffer C/D before adding substrate reagent (R&D Systems). The colorimetric reaction was stopped after 2–10 min by adding 2 M H₂SO₄. Absorbance at 450 nm was measured using a conventional microplate reader (Berthold Technologies).

Pull-Down

CD4⁺ T cells were stimulated with IL-27 (160 ng/ml) and anti-CD3 and anti-CD28 (2.5 $\mu\text{g}/\text{ml}$) during 1 h at 37°C . Cells were lysed by sonication in HKMG buffer (10 mM HEPES (pH 7.9), 100 mM KCl, 5 mM MgCl₂,

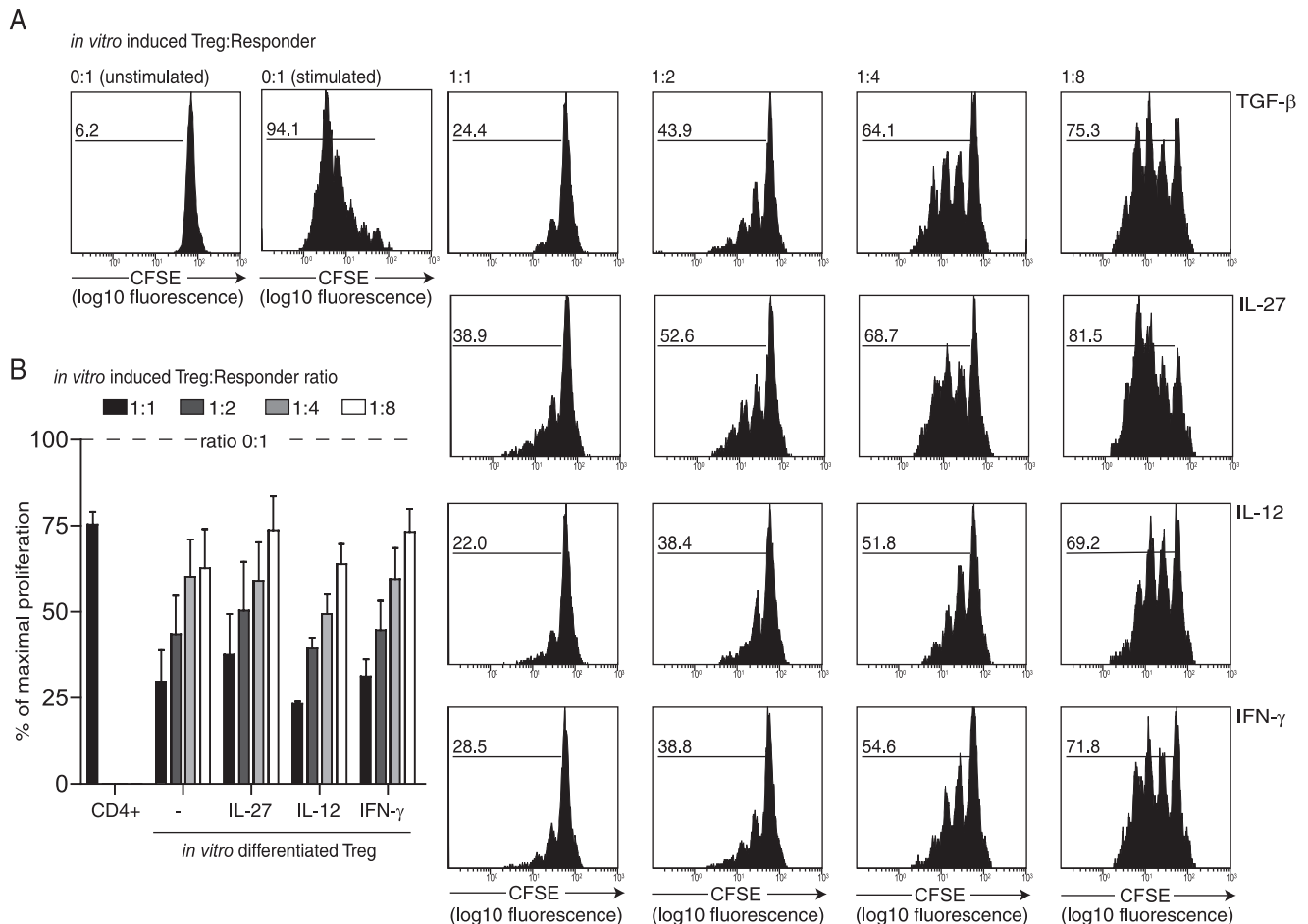


FIGURE 2. Suppressive capacity of iT_{reg} cells cultured with Th1-inducing cytokines. Human CD4⁺CD45RA⁺ T cells were *in vitro* differentiated toward iT_{reg} cells with or without IL-27, IL-12, or IFN- γ . After 10 days of culture, iT_{reg} cells were activated with soluble anti-CD3, autologous irradiated PBMC, and CFSE-labeled CD4⁺ responder T cells in different ratios as indicated. The proliferation of CD4⁺ responder T cells is depicted in A. Data are representative of four independent experiments. B, Percentage of the maximal proliferation. Stimulated CD4⁺ responder T cells without iT_{reg} is set as 100%. Bars show the mean $\pm$ SEM of four independent experiments.

10% glycerol, 1 mM DTT, and 0.5% Nonidet P-40) containing protease inhibitor mixture (Roche Diagnostic). Cell lysates were precleared in streptavidin-agarose beads for 1 h (Amersham Biosciences), then incubated with biotinylated double-stranded oligonucleotides and poly(deoxyinosinic-deoxycytidylic acid) (Sigma-Aldrich) for 4 h at 4°C. DNA-bound proteins were collected with streptavidin-agarose beads for 1 h. The beads were washed three times with buffer HKMG and resuspended in NuPAGE loading buffer (Invitrogen Life Technologies), heated to 55°C for 15 min, and the eluants were separated on a SDS-polyacrylamide gel and identified by Western blotting.

Chromatin immune precipitation (ChIP) assay

The ChIP assay was performed using a ChIP assay kit according to the recommendations of the supplier (Upstate Biotechnology). For precipitation, 3 μ g of Ab against STAT1(M-22), STAT3 (H-190), STAT4 (H-119), STAT5 (H-134) (Santa Cruz Biotechnology), or anti-acetyl histone H4 (Upstate Biotechnology) were used along with a normal rabbit IgG control (Santa Cruz Biotechnology). Primers addressing the FOXP3 promoter region are listed in supplementary Fig. 5. The conventional PCR products were visualized using an ethidium bromide gel. The real-time PCR data were analyzed in relation to the input DNA using the following expression: $E^{-[C_{T(DNA_{input})} - C_{T(DNA_{IP})}]}$.

Transfections and reporter gene assays

T cells were preactivated in complete RPMI 1640 (Life Technologies) containing recombinant human IL-2 (30 ng/ml) and PHA (2 μ g/ml) overnight. For the cotransfection, an amount of 3 μ g of the pGL3-FOXP3 promoter luciferase reporter vector and 2 μ g of the STAT-pCMV-XL4 was added to 6–10 $\times 10^6$ CD4⁺ T cells resuspended in 100 μ l of Nucleofector

solution (Amaxa Biosystems) and electroporated using the T-23 program of the Nucleofector. For the single transfection, 5 μ g of the pGL3-Basic/FOXP3 promoter luciferase reporter vector was used. After a 2-h culture in serum-free conditions and followed by a stimulation as indicated in the figures, the luciferase activity in cell lysates was measured by a dual luciferase assay system (Promega Biotech) according to the manufacturer's instructions in a Berthold Lumat LB 9507 luminometer.

Cloning of the FOXP3 promoter and construction of mutant constructs

The human FOXP3 promoter was cloned into the pGL3 vector (Promega Biotech) to generate the pGL3 FOXP3 -511/+177 as previously described (15). Site-directed mutagenesis in the FOXP3 promoter region was introduced using the QuickChange kit (Stratagene) according to the manufacturer's instructions. The constructs were generated by using the pGL3 -511/+177 as template. Primers used to generate the individual constructs are listed in supplementary Fig. 5.

Statistical analysis

The nonparametric Wilcoxon-matched paired test was used to test statistical significance using the GraphPad Prism software. Values of $p \leq 0.05$ were considered as significant.

Results

FOXP3 expression is regulated by Th1 cytokines

The T cell cytokine receptor-WSX-1 subunit, which is exclusive to the IL-27 receptor, is expressed on CD4⁺ naive T cells (supplementary Fig. 1A). This indicates that IL-27 can directly act on

naive cells. The high expression of its receptor on in vitro-differentiated iT_{reg} cells (supplementary Fig. 1B), as well as on natural T_{reg} cells (29), suggests that IL-27 may regulate T_{reg} cell differentiation and/or function. To elucidate whether IL-27 has a role during iT_{reg} cell differentiation, we cultured human naive $CD4^+$ T cells under conditions leading to the differentiation of iT_{reg} cells (stimulated with anti-CD3/28, anti-IL-4, anti-IL-12, anti-IFN- γ , IL-2, and TGF- β) in the presence or absence of IL-27. Even though cultures in the presence of TGF- β resulted in 54.1 ± 11.8 -fold mRNA induction and $33.4 \pm 6.4\%$ of FOXP3-positive cells, IL-27 significantly further increased this induction to 98.2 ± 19.9 -fold at the mRNA level ($p = 0.0244$) and to $44.1 \pm 6.5\%$ of FOXP3 protein-expressing cells (Fig. 1, A–C). To determine whether IL-27 is the only Th1-inducing cytokine influencing the FOXP3 expression in the presence of TGF- β , we also cultured naive $CD4^+$ T cells under iT_{reg} cell-inducing conditions in the presence of IL-12 or IFN- γ (same as iT_{reg} conditions without anti-IL-12 or anti-IFN- γ , respectively). In the presence of IL-12, TGF- β -induced FOXP3 expression levels were maintained at 56.1 ± 28.9 -fold induction for FOXP3 mRNA and at $29.6 \pm 6.7\%$ of FOXP3-expressing cells. Similar to IL-27, IFN- γ acted as an amplifier of TGF- β -induced FOXP3 expression by inducing 97.3 ± 36.7 -fold FOXP3 mRNA expression ($p = 0.0312$) and $47.7 \pm 8.2\%$ of FOXP3 protein-expressing cells (Fig. 1, A–C).

To further characterize the cell population developing in the presence of Th1-inducing cytokines and TGF- β , we performed IFN- γ intracellular staining (Fig. 1D). Consistent with previous findings (22, 30), the frequency of IFN- γ -producing cells increased upon treatment with IL-27, IL-12, or IFN- γ (23.4 ± 5.1 vs 36.6 ± 10.2 , 56.1 ± 4.7 and $40.3 \pm 9.8\%$, respectively). IFN- γ production was low in the presence of TGF- β ($11.5 \pm 4.5\%$), whereas IL-12-induced IFN- γ production was sustained ($49.9 \pm 12.4\%$). The analysis of the supernatants of the above cell cultures by a Bio-Plex cytokine bead assay confirmed a high amount of IFN- γ production upon culture with IL-12 and TGF- β (supplementary Fig. 2). TGF- β -mediated suppression of the IFN- γ production was not influenced by IFN- γ or IL-27 and IL-4, IL-10, and IL-17 were merely detectable under all conditions (supplementary Fig. 2). Taken together, those findings are ruling out a differentiation into Th1, Th2, or Th17 cells.

The suppressive capacity of iT_{reg} is sustained with IL-27 and IFN- γ

To investigate whether the increased expression of FOXP3 resulted in the acquisition of suppressive capacity, we tested the in vitro-differentiated iT_{reg} cells for their capability to suppress the proliferation of autologous $CD4^+$ responder T cells. All in vitro-differentiated iT_{reg} cells exhibited a suppressive ability in a cell ratio-dependent manner (Fig. 2). In contrast to natural T_{reg} cells that are anergic, iT_{reg} cells still possess the capacity to proliferate upon stimulation. To investigate the effect of this proliferation in the suppression assay, we included stimulated total $CD4^+$ T cells as a control (Fig. 2B). Those cells were stimulated with anti-CD3, anti-CD28, and IL-2 in parallel to the in vitro differentiation of iT_{reg} , and used as suppressors in the suppression assay. The iT_{reg} -mediated suppression of the proliferation of $CD4^+$ responder T cells was higher than the one observed with stimulated total $CD4^+$ T cells. Taken together, these findings suggest the induction of functional iT_{reg} cells in the presence of TGF- β and IL-27 or IFN- γ .

The FOXP3 promoter contains STAT binding sites

Several studies reported a potential role of STAT molecules on the *foxp3* gene regulation (31). STAT3 and STAT5 binding to FOXP3 promoter to the untranslated region and to exons of the *foxp3* gene

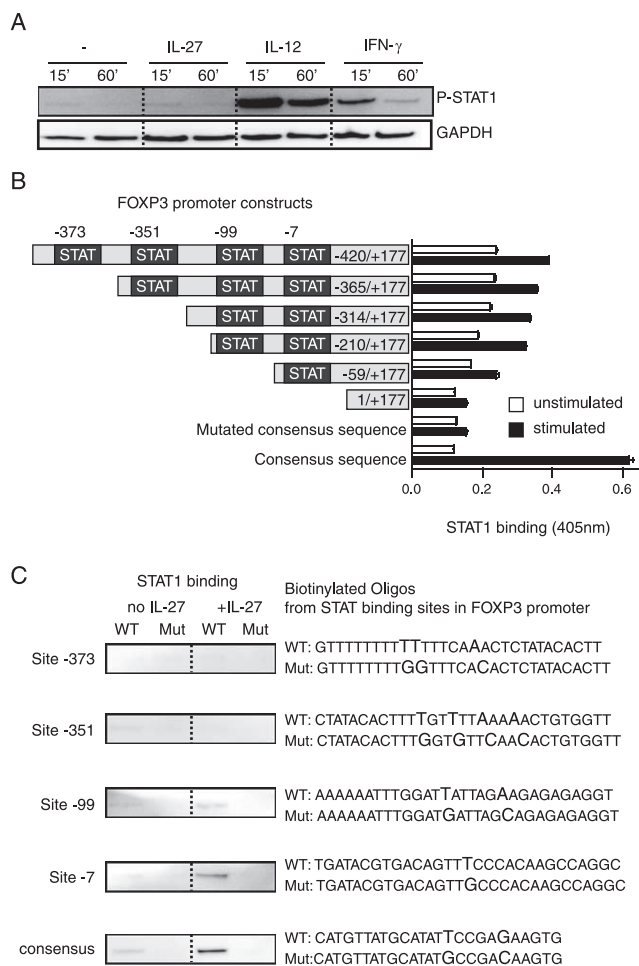


FIGURE 3. STAT 1 binds to the FOXP3 promoter. **A**, Primary human $CD4^+$ T cells were stimulated with anti-CD3/28, IL-2, TGF- β with or without IL-27, IL-12, or IFN- γ for 15 and 60 min. The nuclear extract was analyzed by Western blot for STAT1 Tyr⁷⁰¹ phosphorylation. GAPDH was used as an internal loading control. Data are representative of three independent experiments. **B**, Primary human $CD4^+$ T cells were unstimulated or stimulated with anti-CD3/28, IL-2, TGF- β , and IL-27 for 30 min. The nuclear extract was tested for the binding of STAT1 to the consensus sequence or the mutated consensus sequence and to different lengths of the FOXP3 promoter. Bars show the mean $\pm$ SD of triplicates representative of two independent experiments. **C**, Primary human $CD4^+$ T cells were added to culture with medium or stimulated with anti-CD3/28, IL-2, and TGF- β with or without IL-27 for 30 min. The nuclear extract was tested for the binding of STAT1 to 30-bp probes containing a putative STAT binding site and a STAT-specific consensus sequence wild type (WT) or mutated (Mut) by pull-down analysis. Data are representative of three independent experiments.

have been reported (18, 19, 32). Transcription Element Search Software analysis of the human FOXP3 promoter predicted four putative STAT binding sites at 373, 351, 99, and 7 bp upstream of the TSS. Positions of STAT binding sites within the FOXP3 promoter and primers used in assays are demonstrated in supplementary Fig. 3. Both IL-27 and IFN- γ are known to induce STAT1 Tyr⁷⁰¹ phosphorylation and activation. Since the induction of FOXP3 is dependent on TGF- β signaling, we analyzed whether IL-27- and IFN- γ -induced STAT1 phosphorylation takes place in the presence of TGF- β . Both cytokines induced STAT1 phosphorylation, which enables its DNA binding, whereas IL-12 did not affect this molecule (Fig. 3A).

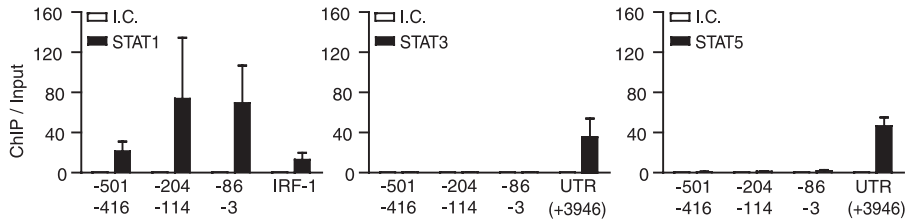


FIGURE 4. STAT1 is the only IL-27-induced STAT member binding the FOXP3 promoter in vivo. Primary human CD4⁺ T cells were stimulated with anti-CD3/28, IL-2, TGF- β , and IL-27 for 30 min. Cells were analyzed by ChIP for STAT1, STAT3, and STAT5 binding to the FOXP3 promoter. The chromatin DNA obtained before (Input) and after immunoprecipitation with anti-STAT Ab or with the respective isotype control was analyzed by real-time PCR with three specific primers pairs for the FOXP3 promoter. The immunoprecipitation with the isotype control (I.C.) is set as 1. Bars show the mean $\pm$ SEM of three independent experiments. URT, untranslated region; IRF-1, IFN regulatory factor 1.

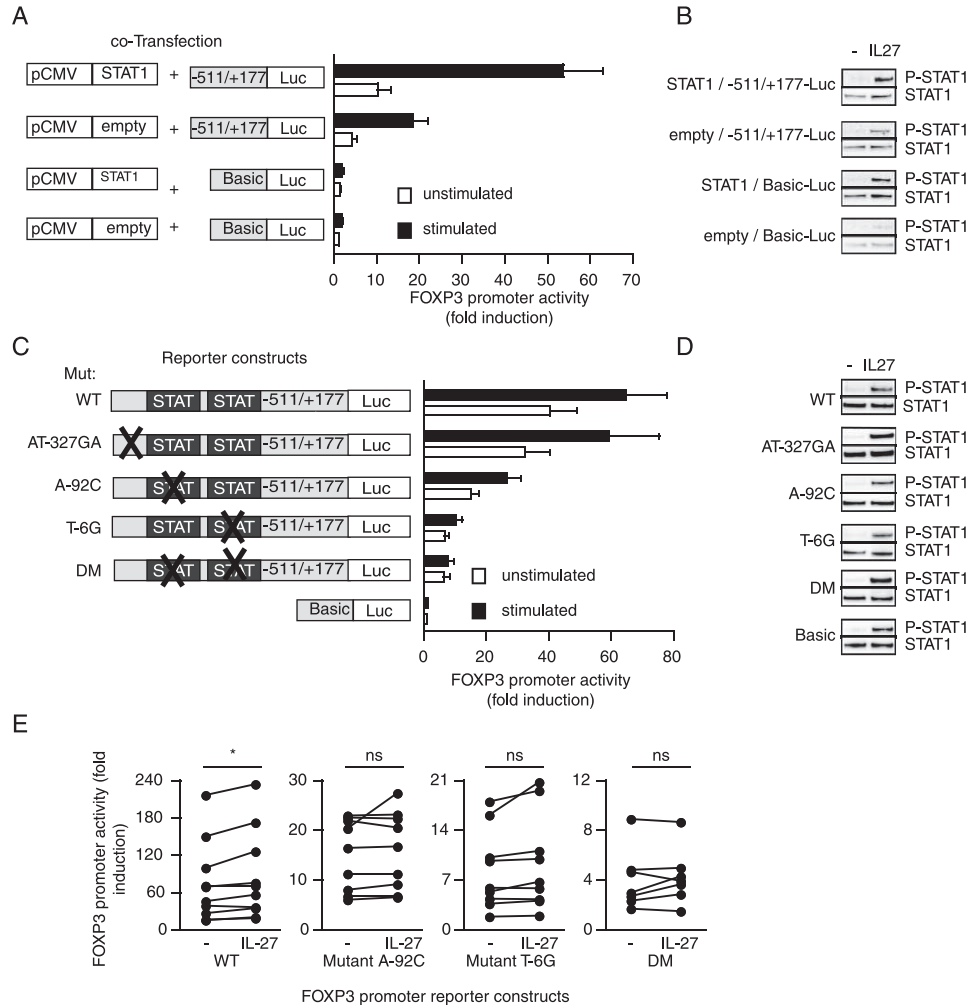
Direct binding of STAT1 to the FOXP3 promoter was investigated in a FOXP3 promoter ELISA analysis. In this assay, oligonucleotides corresponding to different lengths of the FOXP3 promoter were coated on ELISA plates. STAT1 binding was detectable by chemiluminescence following incubation with nuclear extract from IL-27-stimulated CD4⁺ T cells and by using appropriate Abs and detection reagents. STAT1 strongly bound to its specific consensus sequence, but not to the mutated sequence, which validated the specificity of the assay. STAT1 bound the full-length FOXP3 promoter (−420/+177) containing all STAT binding sites. A similar binding was observed in FOXP3 promoter constructs lacking sites at positions −373 and/or −351 bp (−365/+177, −314/+177, −210/+177). However, it was reduced in the

construct lacking the site at position −99 bp (−59/+177) and was totally abolished in the construct lacking the site at position −7 (−1/+177). In agreement with the FOXP3 promoter ELISA results, STAT1 binding was observed to the sites −99 and −7 bp in pull-down analysis using 30-bp probes containing putative STAT binding sites and the surrounding regions in the FOXP3 promoter (Fig. 3C). Together, these data suggest that activated STAT1 can recognize and bind to two of the STAT binding sites in the FOXP3 promoter.

STAT1 binds the FOXP3 proximal promoter

Because IL-27 can also activate other STAT family members, we further tested the implication of the other IL-27-activated STAT

FIGURE 5. IL-27-induced STAT1 regulates FOXP3 promoter activity. Primary CD4⁺ human T cells were preactivated with IL-2 and PHA prior to transfection. **A**, Cells were cotransfected with pGL3 (Basic or FOXP3 −511/+177) and pCMV (empty or STAT1) vectors as indicated and stimulated with anti-CD3/28, IL-2, TGF- β , and IL-27 18 h before luciferase measurement. **B**, The nuclear extract from cells stimulated during 30 min in **A** was analyzed by Western blot for STAT1 Tyr⁷⁰¹ phosphorylation. **C**, Cells were transfected with an empty vector (pGL3 Basic), a WT FOXP3-promoter containing vector, or a mutated (Mut) FOXP3-promoter containing vector and stimulated with anti-CD3/28, IL-2, TGF- β , and IL-27. **D**, The nuclear extract from cells stimulated during 30 min in **C** was analyzed by Western blot for STAT1 Tyr⁷⁰¹ phosphorylation. **E**, Cells transfected in **C**, were stimulated with anti-CD3/28, IL-2, and TGF- β with or without IL-27 during 18 h before measurement for the relative luciferase light units. Unstimulated basic vector is set as 1. Bars show the mean $\pm$ SEM of four to eight independent experiments. *, $p < 0.05$. Data in **B** and **D** are representative of four independent experiments. WT, Wild type; ns, not significant.



molecules (e.g., STAT3 and STAT5) for their ability to bind the putative STAT binding sites within the FOXP3 promoter. A slight binding of STAT3 was also observed to the site at position -7 and even though STAT5 was activated and able to bind the STAT consensus sequence following IL-27 stimulation, there was no apparent binding to any of the probes (supplementary Fig. 4 and data not shown). To clarify the importance of the STAT molecules' binding in regulating *foxp3* gene expression in vivo, we performed a ChIP assay. In primary CD4⁺ naive T cells, only STAT1 binding was clearly detectable to the FOXP3 promoter following stimulation with TGF- β , anti-CD3, anti-CD28, IL-2, and IL-27 for 30 min (Fig. 4). The location of STAT1 binding in vivo was evaluated by ChIP assay using PCR primers amplifying three regions of the FOXP3 promoter ($-501/-416$, $-204/-14$, $-86/-3$). PCR primers for the promoter region of IFN regulatory factor 1 and for the untranslated region of the *foxp3* gene were used as positive control to assess the suitability of the Abs for the ChIP assay. In agreement with the pull-down analysis, STAT1 was binding to the regions in the proximity of the sites at positions -99 and -7 bp ($-214/-114$, $-83/-3$) (Fig. 4). Thus, STAT1 was the only STAT member binding to sites at positions -99 and -7 bp of the FOXP3 proximal promoter in vivo following IL-27 stimulation.

IL-27-induced STAT1 regulates *foxp3* gene expression

Functional significance of STAT1 binding to the FOXP3 promoter was analyzed by FOXP3 promoter luciferase assay using IL-27-stimulated CD4⁺ T cells. The strongest luciferase activity was generated with $-511/+177$ pGL3 FOXP3 promoter reporter plasmid after cotransfection with STAT1 (Fig. 5A), reflecting a role of STAT1 in regulating the FOXP3 promoter activity. The respective contribution of the -99 and -7 bp sites in the STAT-mediated regulation of FOXP3 was examined using mutant constructs. The mutations A-92C and T-6G, which abolished the STAT-binding capacity, greatly decreased the overall activity of the promoter, whereas an unrelated mutation corresponding to a AP-1 binding site (AT-327GA) did not change reporter activity (Fig. 5B). This underlined the importance of those binding sequences in the regulation on the promoter activity. IL-27 slightly increased the transactivation of the FOXP3 promoter luciferase reporter construct ($p = 0.0321$; Fig. 5C). This effect was no longer visible in the mutated constructs, suggesting that IL-27-mediated effect takes place through STAT1 binding to the sites at positions -99 and -7 bp.

IL-27 controls *foxp3* gene expression at the epigenetic level

STAT1 has been shown to be implicated in chromatin remodeling following IFN- γ stimulation (33). A single point mutation that prevented its phosphorylation was sufficient to abolish the chromatin remodeling. The role of IL-27 in histone modification of the FOXP3 promoter was investigated by performing ChIP assays with anti-acetyl histone H4 (Fig. 6). In iT_{reg} cells (cultured for 8 days) histone H4 molecule was acetylated in the $-501/-416$ region of the FOXP3 promoter (Fig. 6). Addition of IL-27 in the culture-induced histone H4 acetylation in the regions near STAT1-binding sequences ($-204/-144$, $-86/-3$). This indicated a role of IL-27-induced STAT1 in regulating the FOXP3 proximal promoter by chromatin remodeling.

Collectively, these findings suggest a potentiation of TGF- β -induced FOXP3 expression in the presence of IL-27 and IFN- γ , while IL-12 remained without effect. Furthermore, we showed that IL-27-activated STAT1 binds to specific elements within the proximal region of the FOXP3 promoter, which increases the transactivation and induces permissive histone modifications of this key region.

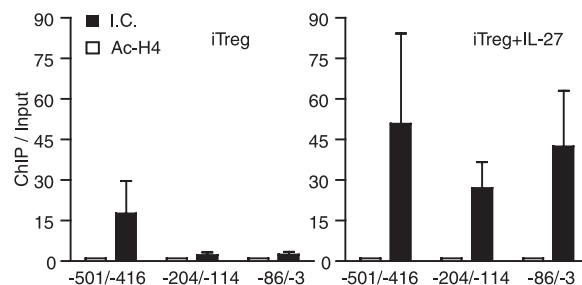


FIGURE 6. IL-27-induced STAT1 regulates histone acetylation. Human CD4⁺CD45RA⁺ T cells were activated with soluble anti-CD3/CD28 and differentiated in iT_{reg}-driven conditions without or with IL-27. After 8 days, cells were analyzed by ChIP for histone H4 acetylation in the FOXP3 promoter. The chromatin DNA obtained before (Input) and after immunoprecipitation with anti-acetyl H4 Ab or with the isotype control (I.C.) was analyzed by real-time PCR with three specific primer pairs for the FOXP3 promoter. The immunoprecipitation with the isotype control is set as 1. Bars show the mean $\pm$ SEM of four independent experiments.

Discussion

By acting directly on chromatin remodeling (34), the transcriptional regulator FOXP3 plays an important role in the early differentiation process of iT_{reg} cells. FOXP3 is up-regulated in naive T cells stimulated by TGF- β to induce iT_{reg} cells. The current study reveals that FOXP3 expression is boosted by IL-27 or IFN- γ costimulation with TGF- β via a mechanism involving the activation and the binding of STAT1 molecule to specific elements within the proximal part of the FOXP3 promoter and by the modulation of histone acetylation of this region.

Costimulation of naive CD4⁺ T cells with TGF- β and IL-27 or IFN- γ induces higher FOXP3 expression than TGF- β stimulation alone and generates potently suppressive iT_{reg} cells. Because naive CD4⁺ T cell differentiation is orchestrated by lineage-specific factors, we anticipated that iT_{reg} cells driven in the presence of IL-27 or IFN- γ would exhibit higher suppressive capacity. However, the proliferative response of cocultured CD4⁺ responder T cells was similar between all IL-27- or IFN- γ -boosted iT_{reg} and only TGF- β -induced iT_{reg} cells. Since posttranslational modifications of FOXP3 influence its function in vivo (35), additional factors modifying the acetylation or phosphorylation of FOXP3 may be required to translate expression differences into higher suppressive functionality.

In the presence of TGF- β , IL-12 promotes the development of proinflammatory cytokine-producing cells to a great extent, suggesting that IL-12-driven differentiation increase the plasticity of TGF- β -mediated iT_{reg} cells. The functional relevance of the plasticity of the *ifn*- γ locus is still unclear, but the switch to the Th1 phenotype has been reported following ectopic expression of T-bet in lineage-committed Th2 cells (36). In contrast to IL-12, both IL-27 and IFN- γ did not modulate IFN- γ expression in recall responses, while effectively promoting TGF- β -mediated iT_{reg} generation. This regulatory property of IL-27 has also been observed in recent studies showing that it acts as an antagonist of T cell-mediated inflammation, as a regulator of Th2 responses, as an inhibitor of Th17 cells development, and as an inhibitor of T cell proliferation (37). However, the mechanisms by which IL-27 exerts its suppressive effects are still unclear. In this study, we provide evidence for a function of IL-27 in potentiating the effect of TGF- β on the induction of FOXP3 and, thus, in promoting the differentiation of iT_{reg} cells.

Although a regulatory role of IL-27 has been demonstrated, the underlying molecular mechanisms are currently controversially

discussed. Murine studies showed that IL-27 inhibits TGF- β -driven induction of FOXP3 and iT_{reg} cell differentiation (38, 39). Interestingly, a clear species-specific function was reported for IL-27 (40). Murine macrophages were shown to be minimally IL-27 responsive, whereas human monocytes are strongly activated by IL-27 in a STAT1-dominant manner (40). This suggests that IL-27-induced-STAT1-dependent effects might be species specific. The equivalence of FOXP3's regulation and function between mice and human is also a matter of debate. Although Foxp3 is exclusive to T_{reg} cell lineage in mice, it can be transiently expressed in activated human T cells (41–43). However, the FOXP3⁺ cells acquire suppressor activity after prolonged cultured (13) and lentivirus-based ectopic expression of FOXP3 is sufficient to generate potent and stable human CD4⁺ T_{reg} cells (44). Even though the regulation of FOXP3 expression differs between humans and rodents, its expression in human CD4⁺ T cells relates to regulatory activity.

Consistent with previously demonstrated STAT1-mediated effect of IL-27 on naive T cell differentiation, we show that IL-27 regulates the induction of the human *foxp3* gene through the activation of STAT1. By binding to the proximal part of the promoter, STAT1 controls the level of transcription of the *foxp3* gene. In addition to STAT1, the pull-down analysis reveals a slight binding of STAT3 to the FOXP3 promoter. STAT1 and STAT3 were suggested to have opposite function (45) and it was recently shown that STAT3 inhibits iT_{reg} development (46). IL-6 is a major activator of STAT3 and it acts in concert with TGF- β to induce the expression of retinoic orphan receptor C2, which leads to Th17 cell differentiation (3, 4). Considering the close relationship between Th17 and iT_{reg} cells, IL-27 might play a critical role in the lineage decision-making between Th17 and iT_{reg} cells. A similar critical function of IL-27 is observed during Th1 and Th2 differentiation. IL-27 significantly increases IFN- γ production, even at low levels of IL-12, during *Leishmania major* infection, whereas it inhibits the expansion phase of the Th2 cells at high levels of IL-4 during *Trichuris muris* infections (23, 47, 48). In the presence of TGF- β , IL-27-induced STAT1 might prevent the inhibitory effect of STAT3 binding to the FOXP3 promoter and, thus, favoring iT_{reg} differentiation.

STAT5 has also been shown to be important for both the development and the maintenance of Treg cells. Constitutive STAT5b activation in Treg cells can rescue their development in the absence of IL-2R signaling (18). Thus, STAT5 was suggested to provide the direct link between the IL-2R signals and the FOXP3 expression through direct binding of STAT5 to several binding sequences within the *foxp3* gene (18, 19, 32). However, IL-2 signaling also results in STAT3 phosphorylation, which has the opposite effect on the *foxp3* gene expression (46). Future research is necessary to clarify how STAT molecules interact with other coactivators to mediate distinctive effects following cytokine stimulation. The IL-27 signaling also results in the activation of STAT5 in addition to STAT1 or STAT3 and is likely to play a critical role in the regulation of the *foxp3* gene by interacting with numerous regulatory binding sequences.

The expression of the *foxp3* gene is also related to its chromatin structure (20, 49). Permissive histone modifications of the FOXP3 promoter, the intronic differentially methylated region 3, and the enhancer at position +2079 to +2198 were reported in T_{reg} cells (16, 20, 49). The histone modifications near the *foxp3* TSS were described as a marker of the inducibility of Foxp3 and correlate with its expression (50). In this study, confirm that histone acetylation of this region promotes FOXP3 expression and reveal that this mechanism is mediated by IL-27. The late increase of FOXP3 following TGF- β stimulation implies the need of numerous intra-

cellular mechanisms to regulate its expression. The rapid binding of STAT1 to the FOXP3 promoter argues that IL-27-induced STAT1 may regulate early events, allowing the recruitment and binding of the other regulatory factors (e.g., NFAT, AP-1, Sp-1) to empower *foxp3* gene transcription.

Our data suggest a new pathway of regulation for FOXP3 induction with permissive epigenetic modification of the proximal promoter by the IL-27-induced STAT1 molecule. The mechanisms involved in regulating FOXP3 in iT_{reg} cells are of considerable interest due to the essential contribution of FOXP3 in preventing autoimmune diseases. Therapies, which aim to augment the number or the function of T_{reg} cells, represent a very promising approach to restoring tolerance in many immune-mediated diseases.

Disclosures

The authors have no financial conflict of interest.

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FIG. 1

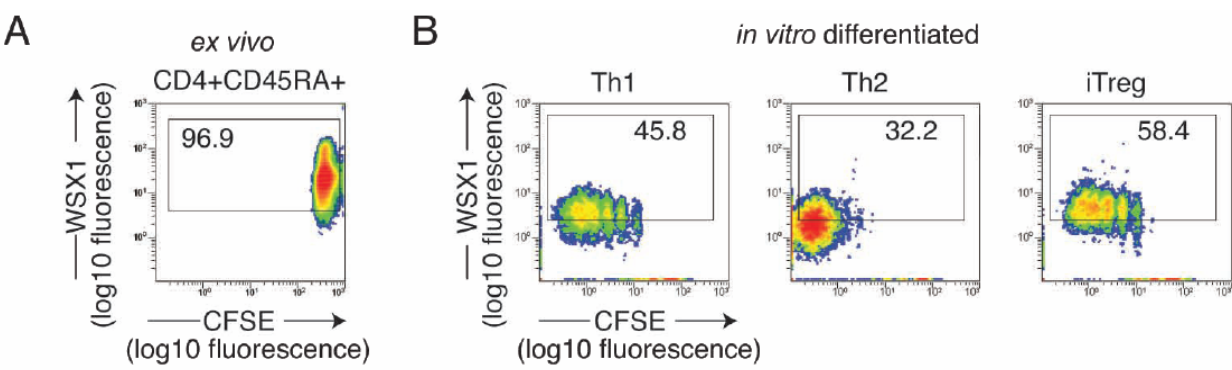


FIG. 2

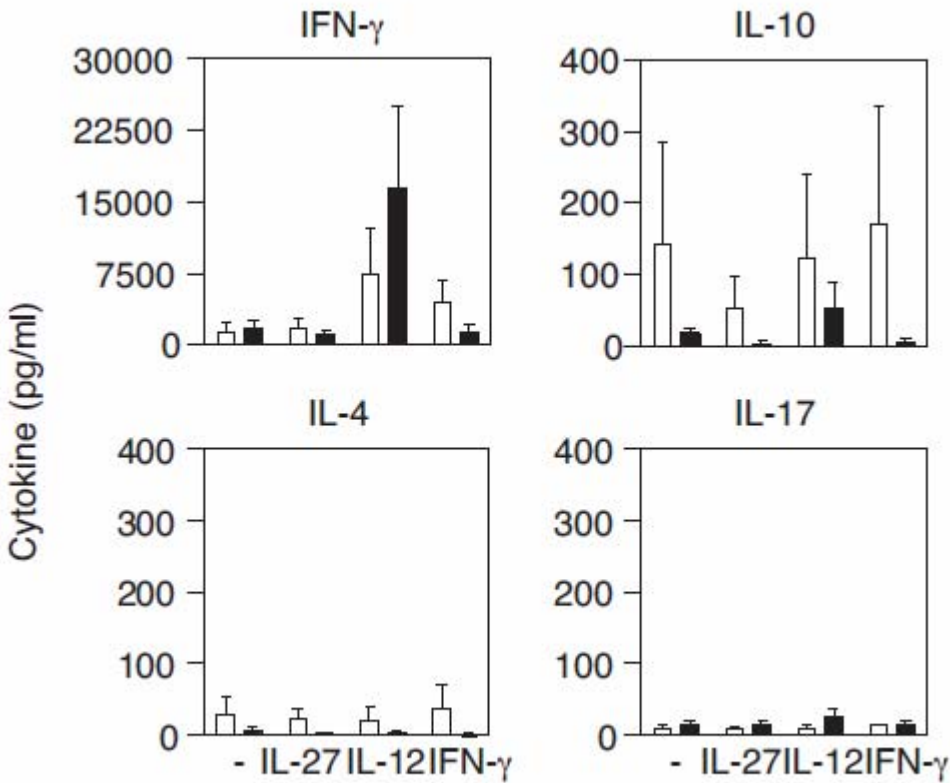


FIG. 3

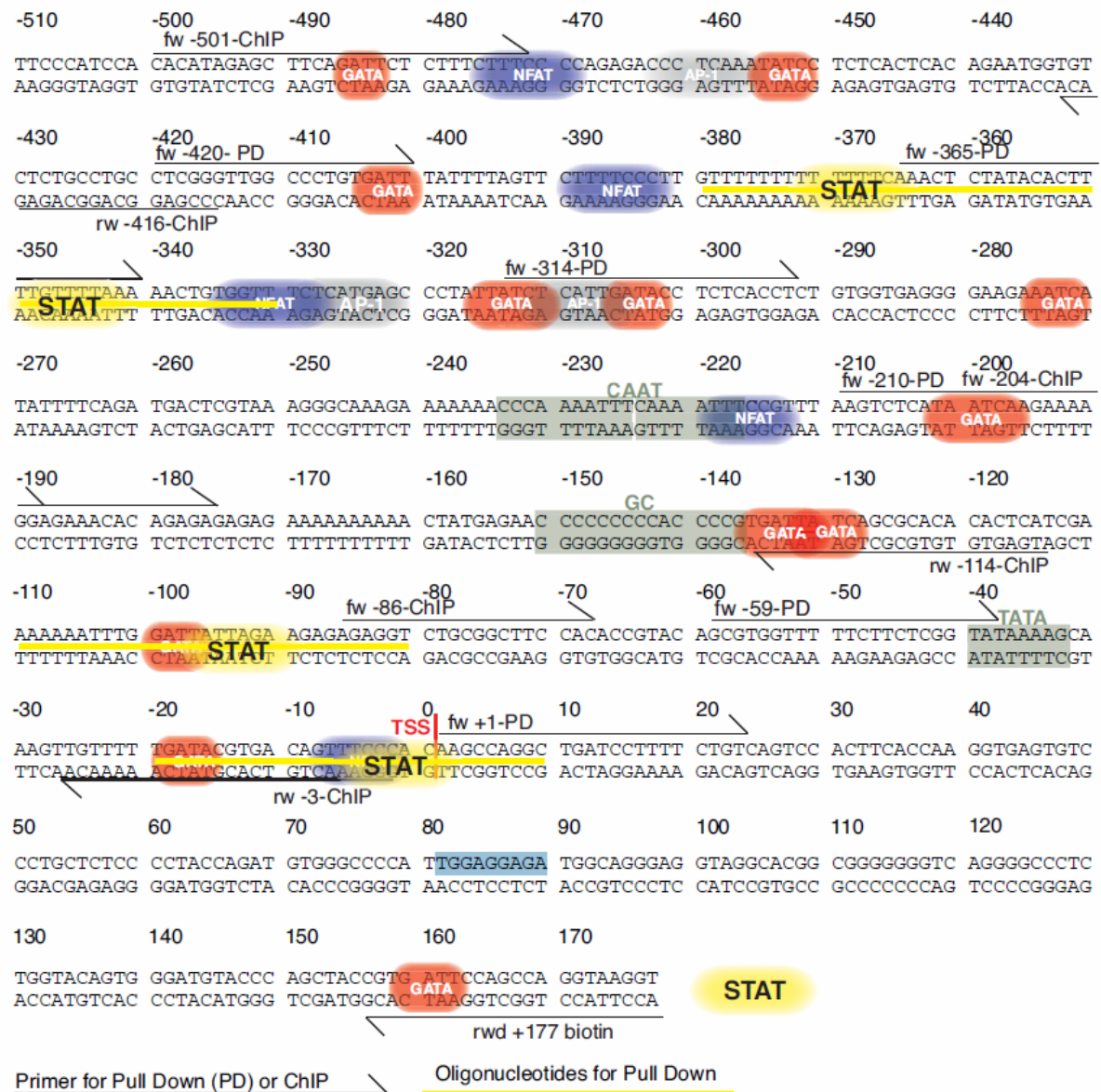


FIG. 4

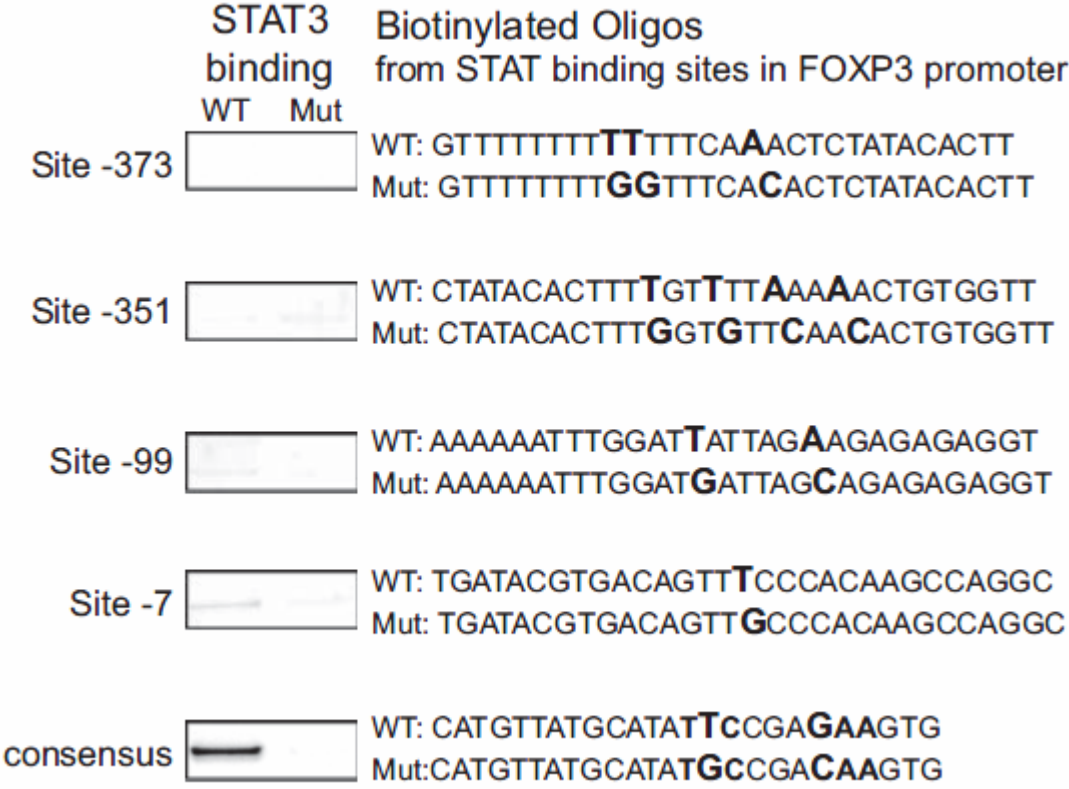


FIG. 5

List of oligonucleotide and primer sequences (5' to 3')

Real Time PCR

FOXP3 sense: GAAACAGCACATTCCCAGAGTTC
FOXP3 antisense: ATGGCCAGCGGATGAG
Elongation Factor 1a sense: CTGAACCATCCAGGCCAAAT
Elongation Factor 1a antisense: GCCGTGTGGCAATCCAAT

Pull Down ELISA

FOXP3 promoter -1/+177 sense: AAGCCAGGCTGATCCTTTTCTGT
FOXP3 promoter -59/+177sense: CGTGGTTTTTCTTCTCGGTA
FOXP3 promoter -210/+177 sense: AGTCTCATAATCAAGAAAAGG
FOXP3 promoter -314/+177 sense: TCTCATTGATACCTCTCACCT
FOXP3 promoter -365/+177 sense: AACTCTATACACTTTTGTAAA
FOXP3 promoter -420/+177 sense: CTCGGGTTGGCCCTGTGATT
FOXP3 promoter antisense: ACCTTACCTGGCTGGAATCACG
STAT consensus sequence WT: CATGTTATGCATATCCGAGAAGTG
STAT consensus sequence M: CATGTTATGCATATGCCGACAAGTG

Pull Down Assay

FOXP3 promoter region (-381/-351) WT: GTTTTTTTTTTTCAAACCTCTATACACTT
FOXP3 promoter region (-381/-351) M: GTTTTTTTTTGGTTTCACTCTATACACTT
FOXP3 promoter region (-361/-331) WT: CTATACACTTTTGTAAAACCTGTGGTT
FOXP3 promoter region (-361/-331) M: CTATACACTTTGGTGTCAACACTGTGGTT
FOXP3 promoter region (-111/-81) WT: AAAAAATTTGGATTATTAGAAGAGAGAGGT
FOXP3 promoter region (-111/-81) M: AAAAAATTTGGATGATTAGCAGAGAGAGGT
FOXP3 promoter region (-21/+11) WT: TGATACGTGACAGTTTCCACAAGCCAGGC
FOXP3 promoter region (-21/+11) M: TGATACGTGACAGTTGCCACAAGCCAGGC
STAT consensus sequence WT: CATGTTATGCATATCCGAGAAGTG
STAT consensus sequence M: CATGTTATGCATATGCCGACAAGTG

ChIP Assay

RT-PCR FOXP3 promoter region (-501/-416) sense: CACATAGAGCTTCAGATTCTCTTTCTTT
RT-PCR FOXP3 promoter region (-501/-416) antisense: CCGAGGCAGGCAGAGACA
RT-PCR FOXP3 promoter region (-204/-114) sense: ATAATCAAGAAAAGGAGAAACACAGAGA
RT-PCR FOXP3 promoter region (-204/-114) antisense: TGAGTGTGTGCGCTGATAATCA
RT-PCR FOXP3 promoter region (-86/+3) sense: AGAGGTCTGCGGCTTCCA
RT-PCR FOXP3 promoter region (-86/+3) antisense: GGAACTGTACGTATCAAAAACAA
RT-PCR IRF-1 promoter sense: CTTCCGCGCTAGCTCTACAACAG
RT-PCR IRF-1 promoter antisense: GCTCCGGGTGGCCTCGGTTCCG
RT-PCR FOXP3 UTR site +3946 sense: TCTTCCTCCGTGGTGTGAT
RT-PCR FOXP3 UTR site +3946 antisense: ATCTGGGCCCTGTTGTCA

Mutagenesis

FOXP3 promoter site -99 sense: GAAAAAATTTGGATTATTAGCAGAGAGAGGTCTGCGGCTTC
FOXP3 promoter site -99 antisense: GAAGCCGCAGACCTCTCTGCTAATAATCCAAATTTTTTC
FOXP3 promoter site -7 sense: GATACGTGACAGTGTCCACAAGCCAG
FOXP3 promoter site -7 antisense: CTGGCTTGTGGGACACTGTACGTATC
FOXP3 promoter site UR sense: TTGTTTTAAAACCTGTGGTTTCTCGAGAGCCCTATTATCTCATTGATACC
FOXP3 promoter site UR antisense: GGTATCAATGAGATAATAGGGCTCTCGAGAAACCACAGTTTAAAAACAA