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FOXP3 Inhibits Activation-Induced NFAT2 Expression in T Cells Thereby Limiting Effector Cytokine Expression


The forkhead DNA-binding protein FOXP3 is critical for the development and suppressive function of CD4+CD25+ regulatory T cells (TREG), which play a key role in maintaining self-tolerance. Functionally, FOXP3 is capable of repressing transcription of cytokine genes regulated by NFAT. Various mechanisms have been proposed by which FOXP3 mediates these effects. Using novel cell lines that inducibly express either wild-type or mutant FOXP3, we have identified NFA1 as an early target of FOXP3-mediated transcriptional repression. NFAT2 is typically expressed at low levels in resting T cells, but is up-regulated by NFAT1 upon cellular activation. We demonstrate that transcription from the NFAT2 promoter is significantly suppressed by FOXP3, and NFAT2 protein expression is markedly diminished in activated CD4+CD25+FOXP3− TREG compared with CD4+CD25− FOXP3+ T cells. Chromatin immunoprecipitation experiments indicate that FOXP3 competes with NFAT1 for binding to the endogenous NFAT2 promoter. This antagonism of NFAT2 activity by FOXP3 is important for the anergic phenotype of TREG, as ectopic expression of NFAT2 from a retroviral LTR partially restores expression of IL-2 in FOXP3+ TREG. These data suggest that FOXP3 functions not only to suppress the first wave of NFAT-mediated transcriptional responses, but may also affect sustained NFAT-mediated inflammatory gene expression through suppression of inducible NFAT2 transcription. The Journal of Immunology, 2009, 183: 907–915.
FOX3+ effector T cells. This suppression is mediated by regulation of NFAT1 binding to the NFAT2 promoter and results in decreased cytokine production that can be overcome by expression of NFAT2 from a heterologous promoter. Inhibition of NFAT2 by FOX3 therefore not only inhibits NFAT-mediated cytokine production but also may play a role in the decision between regulatory and effector cell fates.

Materials and Methods

Generation of tetracycline-inducible HEK293 cell lines

Generation of stable HEK 293 cell lines that express amino-terminal V5-tagged wild-type (aa 2-431) or mutant (aa 2-334, lacking the forkhead domain) FOX3 in response to tetracycline has been previously described (5).

Microarray analysis

RNA sample preparation. Total RNA was isolated from 293 cells line expressing WT or mutant (ΔFKH) FOX3 24 h after the addition of doxycycline (1 μg/ml) to induce expression of the FOX3 protein. RNA was isolated using TRIzol reagent (Invitrogen) per the manufacturer’s protocol. Six independent experiments were completed for each cell line. After purification, RNA concentration was determined with a Nanodrop spectrophotometer, and then quantitatively assessed for degradation using the ratio of 28S:26S (A260/A280) measured with a 2100 Bioanalyzer, Agilent Technologies. All RNA samples used in these experiments had a 260/280 absorbance ratio of 2.0.

Microarray slides. The Human V2.0 Qiagen Operon genome-scale 70-mer oligonucleotide library was printed in the microarray facility of the Oklahoma Medical Research Foundation as previously described (11).

Labeling and hybridization. cDNA was synthesized with direct incorporation of Cy3-dUTP from 2 μg total RNA using Endo-Free reverse transcriptase (Ambion). RNA was mixed with 500 ng of anchored oligo-dT primer, brought to 20 μl volume with DEPC water, heated to 70°C for 10 min, and placed on ice to reach 50°C for 10 min. This was added to a solution containing 2 μl of 10X reverse transcription buffer (Ambion); 0.5 mmole Cy3-UTP; 2.5 mM each of dATP, dCTP, and dGTP; 1.5 mM TTP, 40 U of RNase inhibitor; and 1 μl reverse transcriptase. cDNA was synthesized at 50°C for 2 h using a Gene Amp PCR System 9700 (PerkinElmer Applied Biosystems). Reactions were terminated by adding 5 μl of 1.0 M Tris-HCl (pH 8.0). cDNA was neutralized with 25 μl of 1.0 M Tris·HCl (pH 8.0). cDNA was purified with a Montage 96-well format vacuum system (Millipore).

The cDNA was added to hybridization buffer containing Cot-1 DNA (0.5 mg/ml final concentration), yeast tRNA (0.2 mg/ml), and poly(dA)40–60 (0.4 mg/ml). Hybridization was performed on a Ventana Discovery System for 6 h at 42°C. Microarrays were washed to a final stringency of 0.1× SSC. Fluorescence intensity was determined using ImageGauge software (Ventana Biotechnology).

Normalization. Normalization for differences among experiments was conducted as previously described (11, 12). After data normalization and log-transformation, residuals are created from the control group and the “outliers” (genes with significant variability compared with the group) were excluded with an iterative procedure based on the F-test. The rest, called a “reference group” were used as an internal standard of equity of expression in the respective t-test (see below).

Identification of genes differentially expressed. WT vs ΔFKH 293 cell lines. Analyses include the following. First, selection with a Student’s t-test for replicates using the commonly accepted significance threshold of p < 0.05. Because of the large number of genes present on microarrays, a significant proportion of genes identified as differentially expressed in this manner are expected to be false positive determinations at this threshold level. Second, an associative t-test in which the replicated residuals for each gene of the experimental group are compared with the entire set of residuals from the reference group (defined above). The hypothesis that gene expression in the experimental group, presented as replicated residuals (deviations from averaged control group profile), is distributed similarly to the several thousand members of the normally distributed set of residuals for gene expression in the reference group is tested. The significance threshold is corrected to 1/(number of genes) to make it improbable that false positives arise with p values below the threshold of both the Student t-test and the associative t test are then presented in tables as differentially expressed genes. Third, a distinctive group of selections consisted of uniquely expressed genes. Genes expressed distinctively from background in one group and not distinctively background in another are defined as only in one group expressed genes.

The microarray data discussed in this publication have been deposited in National Center for Biotechnology Information’s Gene Expression Omnibus (13) and are accessible through GEO Series accession number GSE13798 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE13798).

Human T cell purification and transfection

Primary human CD4 T cells were purified from whole blood using the StemCell RosetteSep CD4 enrichment mixture per the manufacturer’s protocol. For some experiments, CD4 T cells were additionally purified into CD25+ and CD25− fractions using anti-CD25 magnetic beads (Dynal/Invitrogen). Beads were detached from the cell using the manufacturer’s protocol. Primary CD4 T cells were transiently transfected either with an empty control vector or with a mammalian expression vector encoding nV5-tagged WT FOX3. Transfections (~50% efficiency, data not shown) were done using an Amaxa Nucleofector (Amaza) as previously described (14).

Quantitative RT-PCR

Total RNA was prepared using Trizol reagent as indicated above. First strand cDNA synthesis was performed using the Omniscript RT Kit (Qiagen) in the manufacturer’s protocol. cDNA levels were quantitated using an iCycler iQ Real-Time PCR System (Bio-Rad) and Platinum SYBR Green qPCR Supermix-UDG (Invitrogen). Amplification was conducted in a total volume of 20 μl for 40 cycles with denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Samples were run in triplicate and their relative expression was determined by normalizing to expression of the housekeeping gene HPRT. Primers: FOXP3: Forward 5′-CTGCCCACACTGCCCCCTAGTC-3′, Reverse 5′-CCATTCTGCCCCAGTGGTTTGGTAG-3′. NFAT2: Forward 5′-TTCCGGCCAGCACTTGAGAG-3′, Reverse 5′-CCGTTGTCAGGGTCTGAAG-3′. HPRT: Forward 5′-GTTGGATAGCAGGCGAGCTTGGT-3′, Reverse 5′-CAAAAGGGAACGTGATAGTCTATAGGC-3′.

Luciferase assays

Unless otherwise indicated, 5 × 10^4 primary human CD4 T cells were cotransfected with 5 μg of the indicated firefly luciferase reporter vector (inducible NFAT2 promoterello (8), 3′X-NFAT, IL-2, or CD154 mRNA stability element; Ref. 15), 100 ng of the pRL-TK renilla luciferase control vector (Promega), and 10 μg of the indicated FOX3 expression vector or empty control vector. Two hours after transfection, cells were divided and half were stimulated with PMA (25 ng/ml)/ionomycin (1.5 μM) while the other half were treated with medium alone for 6 h. Luciferase assays were run using the Dual Luciferase Assay Kit (Promega) per the manufacturer’s recommendations. Luminescence was determined in duplicate with a Lumat 9507 luminometer (Berthold Technologies).

Detection of intracellular NFAT2

Primary human T cell subsets were resuspended in RPMI 1640 complete medium at 10^6 cells/ml and treated either with DMSO (diluent) or PMA (5 nM/ionomycin) (2 μL/h) at 37°C. Cells were stained with allophycocyanin anti-CD69 mAb (clone FN50, BD Biosciences) for 30 min. followed by incubation in FOXP3 Fix/Perm buffer (BioLegend). Cells were washed and resuspended in permeabilization buffer (BioLegend) for 15 min, then intracellular staining was performed with AlexaFluor 488 anti-FOXP3 (Clone 259D, BioLegend) and PE anti-NFAT2 (Clone 7A6, Santa Cruz Biotechnology) for 35–45 min. After washing, flow cytometry was performed on an LSR II flow cytometer (BD Biosciences). Expression of NFAT2 in transduced murine CD4 T cells was assessed by immunoplot analysis using an Ab against murine NFAT2 (Abcam).

Chromatin immunoprecipitation (ChIP) analysis

ChIP assay reagents from Upstate Biotechnology with modifications to the manufacturer protocol as previously described (15, 16). Nuclear proteins and chromatin were cross-linked, sonicated, and extracts from 1 × 10^6 cells were precipitated overnight at 4°C with 10 μg of anti-NFAT1 mAb (clone MA1–025, Affinity Bioreagents), anti-NFAT2 mAb (clone MA3–024, Affinity Bioreagents), anti-Foxp3 mAb (eBiosciences), anti-FLAG Ab (Sigma-Aldrich), anti-V5 mAb (Invitrogen), or control IgG Ab. Proteins and chromatin were cross-linked, sonicated, and extracts from 1 × 10^6 cells were precipitated overnight at 4°C with 10 μg of anti-NFAT1 mAb (clone MA1–025, Affinity Bioreagents), anti-NFAT2 mAb (clone MA3–024, Affinity Bioreagents), anti-Foxp3 mAb (eBiosciences), anti-FLAG Ab (Sigma-Aldrich), anti-V5 mAb (Invitrogen), or control IgG Ab and Protein G agarose. DNA extracted from each precipitate was probed by TaqMan real time PCR (Applied Biosystems) using primers and a probe specific for the human or mouse NFAT2 proximal promoter region (hu-forward 5′-GGG AGTTGTTTTCCCCAGGTCTTAAA-3′, hu-reverse 5′- CGAGTTCGAGGTCCTGTGAGT-3′, or μ-forward 5′-CTGTTTGCGCCCTCGT-3′ and μ-reverse 5′-GAGGACACAGGGACCTGGA-3′).

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**FIGURE 1.** Characterization of inducible FOXP3-expressing cell lines and results of array analysis. A, Quantitative real-time RT-PCR showing the tetracycline inducibility of wild-type (WT) or mutant (ΔFKH) FOXP3 mRNA in the Tet/wtFP3 or Tet/ΔkhFP3 293 cell lines. Each represents three independent experiments. B, Western blot showing protein expression in the cytoplasm and nucleus of each cell line in response to tetracycline. Proteins are tagged at the N terminus with a V5 epitope tag and blot is probed with anti-V5 HRP-labeled Ab. C, Genes differentially expressed in 293 cells expressing wild-type (WT) vs mutant (ΔFKH) FOXP3 protein for 24 h. Only a subset of genes related to immune function, cell signaling, or transcription are shown. Boxed genes demonstrate those identified by others as being similarly differentially expressed in Foxp3+ TREG cells or CD4+ T cells chronically expressing Foxp3. NFAT2 is denoted by an arrow.

Fold differences between specific Ab and isotype control immunoprecipitations were calculated using the formula: \(2^{-\Delta Ct}\), where \(\Delta Ct = Ct_{\text{sample}} - Ct_{\text{control}}\).

**Retroviral transduction and detection of IL-2 and IL-2R**

Murine Foxp3 and NFAT2 cDNAs were amplified from C57BL/6 thymus and cloned into the MSCV-based MIGR1 (NFAT2) and MINR1 (Foxp3) retroviral vectors, as described previously (16). To generate retrovirus, constructs were cotransfected with the pCLeco (Imgenex) helper plasmid into the Phoenix ecotropic packaging cell line. Purified murine CD4+CD25+ T cells were activated with PMA (3 ng/ml), ionomycin (1 μM), and IL-2 (10 U/ml) for 24 h, washed, transduced by spinfection with 29 h Phoenix viral supernatants, and expanded on CD3/28-coated plates (1 ng/ml TGFβ and 10 U/ml IL-2). Cells transduced with NFAT2 were identified by expression of GFP, and cells transduced with Foxp3 were identified by expression of nerve growth factor receptor (NGFR) using flow cytometry. Endogenous Foxp3 expression by TREG was detected using a PE-conjugated anti-Foxp3 Ab (eBiosciences), and IL-2 receptor expression was detected using an allophtocyanin-conjugated Ab against CD25 (BD Biosciences). All cells were rested for 18 h in medium, and restimulated with PMA (3 ng/ml) and ionomycin (1 μM) or plate-bound anti-CD25/28 Ab (2 μg/ml). For intracellular detection of IL-2, cultures were restimulated in the presence of monensin for 5 h, fixed, permeabilized, and stained with an allophtocyanin-conjugated anti-IL-2 Ab (BD Biosciences), IL-2 protein was quantified by the mean fluorescence intensity (MFI) of the positive cells as a function of GFP (for NFAT2) or NGFR (for Foxp3) reporter gene expression, as previously described by Wu et al. (6). Secretion of IL-2 into the culture supernatant by transduced cells (50,000 per well) activated for 8 h on CD3/28-coated plates was measured using a standard IL-2 ELISA kit (eBioscience).

**Results**

**Identification of NFAT2 as a target of FOXP3-mediated suppression**

To identify genes regulated at early time points after introduction of FOXP3, we generated cell lines that inductively express either WT or mutant (ΔFKH) FOXP3 in response to tetracycline. The ΔFKH mutant is truncated at aa 334 and lacks the forkhead DNA-binding domain similar to the nonfunctional mutant Foxp3 protein expressed by the Scurfy mouse (5, 17). Both HEK 293 and Jurkat T cell lines were generated but despite multiple attempts using various approaches, the Jurkat T cell lines demonstrated minimal induction of WT FOXP3 mRNA or protein in response to tetracycline and therefore could not be used (data not shown). In contrast, the HEK 293 cell lines expressed no detectable FOXP3 protein at baseline but increased expression as much as 30-fold upon induction with tetracycline (Fig. 1A). As expected, the induced WT FOXP3 protein was constitutively localized to the nucleus whereas a mutant lacking the forkhead DNA-binding domain (ΔFKH), which also contains the nuclear localization sequence, was primarily localized in the cytoplasm (Fig. 1B) (3, 5).

Array analysis was performed as described in Materials and Methods using RNA prepared from HEK 293 cells induced to express either WT or ΔFKH FOXP3 for 24 h. To identify FOXP3-regulated genes, the gene expression profile of cells expressing WT FOXP3 was compared with those expressing ΔFKH FOXP3. Short-term expression of WT FOXP3 in our cell lines led to more genes being induced than suppressed (909 vs 338 respectively), similar to recent observations comparing murine T cells stably expressing Foxp3 vs those lacking Foxp3 expression (18, 19). The relevance of using this non-T cell line to study genes regulated by FOXP3 is demonstrated by the observation that several immune-associated and T cell specific genes are targets of FOXP3 in this system (Fig. 1C). These include several genes recently identified by other investigators as being differentially expressed in Foxp3+ TREG cells vs Foxp3− effector T cells (Fig. 1C) (18–23).

One of the most interesting among these gene targets is the transcription factor NFAT2(NFATc/NFATc1), which was suppressed by WT FOXP3 in our cell lines and similarly has been observed to be differentially down-regulated in Foxp3+ vs Foxp3−
To determine whether FOXP3 exerts its suppressive effect on NFAT2 expression through transcriptional repression of the NFAT2 promoter, primary human CD4+ T cells were transfected with luciferase reporter constructs containing the inducible NFAT2 promoter, a 3× NFAT binding site, the IL-2 promoter, or the CD154 mRNA stability element (used as a transcriptional negative control). For each reporter plasmid, cells were transfected with the indicated expression vectors, stimulated with PMA/ionomycin, and evaluated for induction of luciferase expression. As previously described, WT FOXP3 represses inducible transcription from the IL-2 promoter by >90%, whereas the ΔFKH mutant has no effect on transcriptional activation (Fig. 2B) (3–6). Similarly, WT FOXP3 also exerts a marked suppressive effect on transcriptional activation from isolated NFAT DNA-binding sites (3× NFAT) or from the inducible NFAT2 promoter. In contrast to the IL-2 promoter however, ΔFKH FOXP3 led to some repression of the 3× NFAT and NFAT2 promoter constructs (an average of 27.1 and 41.9%, respectively) (Fig. 2B). The reason for these differences may have to do with the complexity (number of binding sites for other transcription factors that may be activated simultaneously, etc.) of the isolated promoter fragment included in the particular luciferase constructs used. PMA/ionomycin-induced stabilization of luciferase by the CD154 mRNA stability element is not dependent on NFAT and was unaffected by either WT or ΔFKH FOXP3 proteins as predicted. Thus, FOXP3 was capable of inhibiting NFAT2 promoter transcriptional activity.

To determine whether the effect of FOXP3 on NFAT2 mRNA production ultimately equates to differences in NFAT2 protein expression in T cells, we studied primary human CD4+ FOXP3+ and CD4+ FOXP3− T cells for the ability to induce NFAT2 in response to PMA/ionomycin treatment. Primary human CD4+ T cells were isolated from whole blood by negative selection, divided into CD25+ and CD25− populations, and treated for 2.5 h with PMA/ionomycin or DMSO (diluent) before measurement of NFAT2 protein by flow cytometry. Cells were simultaneously stained for FOXP3, NFAT2, and for cell surface expression of the activation marker, CD69. Similar to the observation that cells expressing WT FOXP3 induce less NFAT2 mRNA upon activation, the CD25+ FOXP3+ cells express less NFAT2 protein in response to PMA/ionomycin than the CD25− FOXP3− cells (Fig. 2C). The FOXP3+ cells consistently had slightly higher basal NFAT2 staining than the FOXP3neg T cells but expressed significantly less NFAT2 than the FOXP3− cells with PMA/ionomycin treatment (Fig. 2C). This was consistent based upon the fold change in MFI in four experiments using three different normal donors (Fig. 2D).

In each case, activation of the pooled cells was verified by an increase in CD69 expression in the FOXP3+ population. Due to the early time point, not all of the cells had increased CD69 expression but NFAT2 induction was similar in both CD69+ and CD69− cells (data not shown) so both populations were included in the analysis (Fig. 2C). Thus, FOXP3 expression represses NFAT2 transcription via the inducible NFAT2 promoter resulting in decreased NFAT2 protein expression.

**NFAT proteins bind to the inducible promoter but not the first intronic enhancer of the NFAT2 gene in primary human CD4+ T lymphocytes**

As previously shown in vitro, NFAT2 transcription following T cell activation is markedly enhanced by the binding of NFAT1 to a promoter region upstream of the first exon of NFAT2, termed the P1 region (8, 9). It has also been shown that in the developing heart, a second region (termed P2) within the first intron acts as a transcriptional enhancer and is required for the expression of an alternative isoform of NFAT2 (24). To confirm that the P1 region...
P1 region of the NFAT2 promoter compared with the precipitation done with isotype control IgG. Similarly, NFAT2 binding to this region following activation was also found to be significantly increased (>7-fold) over unstimulated cells, consistent with an autoregulatory function of NFAT2 (9). In contrast, there was no appreciable binding of NFAT1 or NFAT2 to the P2 region within the first intron in unstimulated or stimulated cells (data not shown). As a positive control for the ChIP assay, we also quantitated NFAT1 binding to the IL-2 promoter and found it to be significantly increased following T cell activation with PMA/ionomycin (data not shown). Therefore, NFAT proteins bind to the proximal P1 NFAT2 promoter in vivo following CD4+ T cell activation.

FOXP3 binds to the P1 region of the NFAT2 promoter and inhibits binding of NFAT1

Because NFAT1 is important for activation-induced transcription of the NFAT2 gene (8, 9), we used ChIP to determine whether FOXP3 represses NFAT2 gene transcription by modulating the binding of NFAT1 to the NFAT2 P1 promoter. Interestingly, a consensus forkhead binding site like that predicted to be bound by FOXP3 (6, 18) was identified overlapping the NFAT1 site in the P1 region of the NFAT2 promoter (Fig. 3A). V5-tagged WT FOXP3 was introduced into primary human CD4+ T cells by Amaxa transfection of a plasmid encoding this cDNA. Expression of FOXP3 resulted in a marked decrease of activation-induced NFAT1 binding to the NFAT2 proximal promoter (Fig. 3C). Moreover, ChIP with an anti-V5 epitope Ab demonstrated evidence for V5-tagged FOXP3 binding to the proximal NFAT2 promoter in these same cells (Fig. 3C). This suggests that FOXP3 abrogates NFAT1 binding to the proximal NFAT2 promoter, thus inhibiting activation-induced NFAT2 transcription, in human CD4+ T cells. Interestingly, FOXP3 does not affect inducible binding of Egr-1, an unrelated transcription factor that binds to the NFAT2 promoter (Fig. 3D).

To corroborate the ability of FOXP3 to bind to the NFAT2 promoter, murine FOXP3 was introduced into primary murine CD4+ T cells by retroviral transduction. Because the NFAT1 binding site sequence in the proximal NFAT2 promoter is identical between mouse and man (Fig. 3A), we postulated that the FOXP3 binding site may also be preserved. Following anti-CD3/anti-CD28 mAb activation of murine CD4+ T cells transduced with FOXP3, we observed a marked increase in FOXP3 binding to the proximal mNFAT2 promoter by ChIP assay (Fig. 3D). By comparison, no FOXP3 bound the mNFAT2 promoter in the absence of T cell activation or in T cells transduced with MIGR1 control retrovirus (Fig. 3D).

To ensure that binding of FOXP3 to the NFAT2 promoter is not simply a consequence of ectopic FOXP3 expression, we evaluated binding of native Foxp3 to the NFAT2 promoter using in vitro generated Foxp3-expressing Treg. We and others have previously shown that TGFβ stimulation results in a population of Foxp3 expressing T cells with regulatory function analogous to natural Treg cells (25–28). Purified CD4+CD25+ murine T cells were cultured in vitro with immobilized anti-CD3 and anti-CD28 Ab for three days in the presence or absence of TGFβ. Foxp3 expression was specifically induced by TGFβ in 50–60% of CD4+ T cells in these cultures (data not shown). ChIP analysis demonstrated a marked increase in Foxp3 binding to the NFAT2 promoter upon stimulation in T cells expanded in the presence of TGFβ (Fig. 3E). By comparison, T cells expanded without TGFβ showed no appreciable increase in Foxp3 binding to the NFAT2 promoter (Fig. 3E). Thus, in generating three different FOXP3 expressing populations of primary CD4+ T cells, from both mouse and human, FOXP3 was demonstrated to bind the NFAT2 proximal promoter contained in the NFAT2 promoter luciferase vector (8) is the primary site of action of NFAT1 in human T cells, in vivo ChIP assays were performed in unstimulated and stimulated primary human CD4+ T lymphocytes using either a control Ab or Abs to NFAT1 or NFAT2. As shown in Fig. 3B, stimulation of cells with PMA/ionomycin induces >8-fold binding of NFAT1 protein to the
in vivo upon T cell activation. Moreover, coincident with FOXP3 binding to the NFAT2 proximal promoter in vivo, NFAT1 engagement of the NFAT2 P1 proximal promoter was disrupted.

_Ectopic NFAT2 expression restores IL-2 production in Foxp3<sup>+</sup> T<sub>REG</sub>_n

To determine whether the suppression of NFAT2 plays a causal role in decreased effector cytokine expression by T<sub>REG</sub>, we expressed murine NFAT2 from a heterologous promoter in CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>REG</sub> using a retroviral transduction approach. Purified CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>REG</sub> were mock transduced (first and second panels in A and B) and CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>REG</sub> were mock transduced (first and second panels in A and B), transduced with empty MIGR1 vector (third panel in A and B), or transduced with MIGR1-encoding murine NFAT2-IRES-GFP (last panel on right) and rested cultures were stained for Foxp3 (A) and CD25 (data not shown). Transduced cells were cultured in medium (top panels in B), or restimulated with plate-bound CD3/28 Ab (middle panels in B and in C) or PMA and ionomycin (bottom panels in B), and IL-2 production was assessed by intracellular staining as a function of GFP reporter gene expression (B) or by ELISA (C). In a separate approach, murine CD4<sup>+</sup>CD25<sup>+</sup> T cells were transduced with Foxp3-IRES-NGFR vector alone (x-axis of left panel in D), NFAT2-IRES-NGFR alone (y-axis of left panel in D), or Foxp3-IRES-GFP plus NFAT2-IRES-NGFR in combination (right panel in D). In the left panel, singly transduced cells were mixed before flow cytometry to demonstrate that each population of cells could be spectrally separated. The transduced cells depicted in D were restimulated with plate-bound anti-CD3/28 Ab, and IL-2 production was measured by intracellular staining with an allophycocyanin-conjugated Ab. The graph in E shows the MFI of IL-2 staining as a function of GFP (NFAT2) or NGFR (Empty vector, Foxp3, and Foxp3 plus NFAT2) reporter MFI. Results are representative of three experiments.

CD4<sup>+</sup>Foxp3<sup>+</sup>CD4<sup>+</sup> T cells that had been mock-transduced and restimulated under the same conditions were able to produce IL-2 (Fig. 4B, left middle panel), and secreted more than 15 ng/ml into the supernatant (Fig. 4C, right). This differential was even greater in response to PMA/ionomycin stimulation, where 70% of conventional CD4<sup>+</sup> T cells were IL-2-positive, but the frequency of IL-2 producers among mock- or empty vector-transduced T<sub>REG</sub> remained at 4–5% (Fig. 4B, bottom panels). The frequency of NFAT2-transduced T<sub>REG</sub> capable of producing IL-2 was increased by 1.4-fold compared with empty vector-transduced T<sub>REG</sub> stimulated through CD3/28 (Fig. 4B, middle panels), and almost 2-fold in response to P/I stimulation (Fig. 4B, bottom panels). This increase in IL-2 production was only observed in the GFP<sup>+</sup> (transduced) cells (Fig. 4B). However, this mild increase in the frequency of IL-2 producers was accompanied by a nearly 20-fold increase in the amount of IL-2 secreted into the supernatant by NFAT2-transduced T<sub>REG</sub>, suggesting that NFAT2 controls not only the frequency of T<sub>REG</sub> capable of producing IL-2, but also strongly regulates the amount of IL-2 produced by an individual cell. Simultaneous staining of Foxp3 and intracellular IL-2 confirmed a marked
increase in IL-2 production by Foxp3<sup>+</sup> T<sub>REG</sub> transduced with NFAT2 and confirmed that the observed increase in IL-2 production was not simply derived from contaminating non-T<sub>REG</sub> cells in the culture (supplemental Fig. 1).<sup>7</sup> Ectopic expression of NFAT2 did not influence IFN-γ gene expression by T<sub>REG</sub> (data not shown), but did lead to increased expression of IL-2α (CD25). This could be a direct effect of NFAT2 on transcription of the il2α gene, or more likely is the result of enhanced autocrine IL-2:IL-2R signaling in these cells, which is known to augment CD25 expression.

The results above show that forced expression of NFAT2 can clearly increase IL-2 production by T<sub>REG</sub>, but not to the levels that are produced by conventional CD4<sup>+</sup> T cells. This implies, not surprisingly, that NFAT2-independent mechanisms also exist in these cells to limit inflammatory cytokine gene expression. To address whether NFAT2 can influence Foxp3 function outside of the T<sub>REG</sub> lineage, conventional CD25<sup>+</sup>Foxp3<sup>+</sup>CD4<sup>+</sup> T cells were transduced with Foxp3 alone, NFAT2 alone, or cotransduced with Foxp3 and NFAT2. Cells were then activated with anti-CD3/28 and transcription of the Il2ra gene, or more likely is the result of enhanced autocrine IL-2:IL-2R signaling in these cells, which is known to augment CD25 expression.

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Coexpression of NFAT2 with Foxp3 was sufficient to restore IL-2 production to levels above those observed in cells transduced with an empty vector control or with Foxp3 alone (Fig. 4E). Similar trends were also observed for IL-2 secretion as measured by ELISA (data not shown). Interestingly, IL-2 expression levels in the cotransduced cells remained lower than those observed in cells transduced with NFAT2 alone (Fig. 4E). This suggests that in addition to limiting NFAT2 protein expression, Foxp3 exerts additional suppressive effects on NFAT2 activity (see Fig. 5). Similar to IL-2, we observed that IFN-γ production was also repressed in CD4<sup>+</sup> T cells transduced with Foxp3 alone, and unlike in natural Treg, was likewise restored by ectopic expression of NFAT2 (data not shown). Therefore, restoration of NFAT2 was sufficient to overcome repression of effector cytokine gene expression mediated by Foxp3.

### Discussion

The role of FOXP3 as a transcriptional regulator was recognized at the time the gene was cloned due to its notable sequence homology to the forkhead family of DNA binding proteins, many of which play critical roles in developmental processes (29). It was initially implicated exclusively as a transcriptional repressor (3), but recent genome-wide screens demonstrated that among direct binding targets of FOXP3, more genes were induced than repressed (16, 18, 19, 30). The specific factors that determine whether FOXP3 enhances or represses transcription have not yet been identified, but an association has been made with differential chromatin remodelling at targets that are differentially regulated (16).

Because FOXP3 has the unique capacity to confer a regulatory phenotype upon CD4<sup>+</sup> T cells, we are interested in the genetic program initiated at early time points after FOXP3 expression. To evaluate immediate early and early gene transcription events regulated by FOXP3, we have used a novel HEK 293-based cell system that offers inducible expression of either WT or mutant (ΔFKH) FOXP3. Using this novel cell system, a number of genes regulated by FOXP3 were identified. Despite the use of HEK 293 cells, several T cell-specific genes were identified as targets of FOXP3-mediated regulation and many of these correlate with genes identified by others studying gene expression profiles in CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> vs CD4<sup>+</sup>CD25<sup>L</sup> T cells (18–22).

One of the genes identified as a target of FOXP3-mediated suppression using our approach was the transcription factor NFAT2, a member of the nuclear factor of activated T cell family of transcriptional regulators (31). This was consistent with the findings of other groups studying differential gene expression profiles in Foxp3<sup>+</sup> and Foxp3<sup>L</sup> T cells (18, 23). NFAT2 is expressed at low levels in resting T cells but is markedly induced upon T cell activation, to augment and sustain NFAT-regulated gene transcription, which is important for the development and function of effector T cells (10). Induction of NFAT2 upon T cell activation is mediated primarily by constitutively expressed NFAT1 (8, 9).

We have demonstrated that FOXP3 suppresses the activation-induced expression of NFAT2 mRNA and protein in primary human T cells, and this appears to occur via inhibition of transcription mediated by the proximal NFAT2 P1 inducible promoter (Fig. 2, A–C). Chromatin immunoprecipitation using Abs to NFAT1 showed inducible binding of NFAT1 to the NFAT2 P1 promoter.

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<sup>7</sup>The online version of this article contains supplemental material.
upon cellular activation (Fig. 3B). This binding was markedly decreased in the presence of FOXP3, suggesting that expression of FOXP3 abrogates binding of NFAT1 to the NFAT2 promoter (Fig. 3C). Concomitant with the loss of NFAT1 binding, we observed a marked increase in FOXP3 binding to the NFAT2 P1 promoter upon T cell stimulation (Fig. 3C). Enhanced binding of FOXP3 to other promoters upon T cell activation has been recently reported (16, 18). The abrogation of NFAT1 binding in the presence of FOXP3 is however unique compared with the mechanism described for the IL-2 promoter where FOXP3 is thought to bind to DNA immediately adjacent to NFAT, and to directly interact with NFAT1 to modulate transcription (6, 32). There are differences in the NFAT-binding sites present in the NFAT2 P1 promoter and the well characterized −280 site in the IL-2 promoter (Fig. 3A) (6). These sequence variations may account for the differences observed in binding however it is not yet clear that this is the case. In the IL-2 site and other NFAT binding sites identified by Wu et al. (6), the core binding sequences for NFAT and FOXP3 are adjacent and in almost all cases separated by ~2–4 base pairs (Fig. 3A). In contrast, the putative FOXP3 binding site in the NFAT2 promoter directly overlaps the core NFAT binding element and is in the opposite orientation (relative to NFAT) compared with the site in the IL-2 promoter (Fig. 3A) (6). This arrangement may prevent both proteins from being able to bind DNA simultaneously or may alter the orientation of interacting surfaces that are important for FOXP3:NFAT cooperation on other promoters. It is unlikely that FOXP3 binds elsewhere within the P1 promoter region because the promoter is highly G/C-rich (8) and there are no other sequences that even loosely match the predicted A/T-rich forkhead consensus sequence bound by FOXP3 (6, 18). Thus, FOXP3 appears to inhibit activation induced NFAT2 transcription via competitive binding with NFAT1 at the proximal NFAT2 P1 promoter.

Along these lines, the restoration of IL-2 and IFN-γ production mediated by coexpression of NFAT2 with FOXP3 suggests that NFAT proteins may directly compete with FOXP3 to regulate the NFAT2 P1 promoter, possibly by shifting the balance of promoter occupancy. Alternatively, longstanding evidence obtained by single cell analysis suggests that activated NFAT proteins must exceed a certain threshold level before they can initiate transcription (33). FOXP3 may therefore exert its effects on some NFAT-driven promoters by limiting the amount of activated NFAT available, thereby maintaining levels below the threshold required for transcriptional activation. The 2- to 3-fold over-expression of NFAT2 in this transduction model (data not shown) may breach the threshold level of NFAT proteins required to increase NFAT-driven gene transcription. FOXP3 may act differently on various promoters to modulate transcription and its effect may be dependent on other proteins that bind concurrently with FOXP3. Nevertheless, overexpression of NFAT2 is capable of overcoming the repressive effects of FOXP3 on cytokine production.

Because NFAT proteins are master regulators of CD4+ T cell derived cytokine transcription, it teleologically makes sense for NFAT proteins to be a major target for down-modulating immune responses and for maintaining an anergic state (34). FOXP3 may be particularly effective because of its ability to inhibit NFAT-mediated transcription at two levels (Fig. 5). First, FOXP3 inhibits initial NFAT mediated immune responses by cooperatively binding to cytokine promoters with NFAT where it is thought to actively suppress transcription (6). Second, it suppresses the second wave of NFAT activation by inhibiting the up-regulation of NFAT2 in response T cell activation thereby maintaining the level of activated NFAT proteins below that required for transcriptional activation (33). NFAT2, in particular, seems to be an attractive target of inhibition in FOXP3-expressing T<sub>REG</sub> cells because in addition to its role in maintaining cytokine transcription, NFAT2 may also be critical for lineage commitment to CD4<sup>+</sup> T cell effector function (10). Thus, by inhibiting NFAT2 transcription directly via the NFAT2 promoter, FOXP3 is able to suppress cytokine expression and effector cell development simultaneously.

Disclosures
The authors have no financial conflict of interest.

References


