Inhibition of Suppressive T Cell Factor 1 (TCF-1) Isoforms in Naive CD4⁺ T Cells Is Mediated by IL-4/STAT6 Signaling*

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The Wnt pathway transcription factor T cell factor 1 (TCF-1) plays essential roles in the control of several developmental processes, including T cell development in the thymus. Although previously regarded as being required only during early T cell development, recent studies demonstrate an important role for TCF-1 in T helper 2 (Th2) cell polarization. TCF-1 was shown to activate expression of the Th2 transcription factor GATA-binding protein 3 (GATA3) and thus to promote the development of IL-4-producing Th2 cells independent of STAT6 signaling. In this study, we show that TCF-1 is down-regulated in human naive CD4⁺ T cells cultured under Th2-polarizing conditions. The down-regulation is largely due to the polarizing cytokine IL-4 because IL-4 alone is sufficient to substantially inhibit TCF-1 expression. The IL-4-induced suppression of TCF-1 is mediated by STAT6, as shown by electrophoretic mobility shift assays, chromatin immunoprecipitation, and STAT6 knockdown experiments. Moreover, we found that IL-4/STAT6 predominantly inhibits the shorter, dominant-negative TCF-1 isoforms, which were reported to inhibit IL-4 transcription. Thus, this study provides a model for an IL-4/STAT6-dependent fine tuning mechanism of TCF-1-driven T helper cell polarization.

The transcription factors TCF-1 (T cell factor 1) and LEF-1 (lymphoid enhancer-binding factor 1) are downstream effectors of the canonical Wnt pathway, also known as the Wnt/β-catenin signaling pathway (1, 2). TCF-1 and LEF-1 have a dual function in gene regulation that is determined by their associated co-factors. In the absence of Wnt ligand, the co-factor β-catenin is targeted for proteasomal degradation, and the Wnt pathway transcription factors TCF-1 and LEF-1 repress target gene expression by interacting with repressors that belong to the Groucho-related family (3, 4). Transient activation of the Wnt cascade allows β-catenin to escape proteasomal degradation. Stabilized β-catenin then translocates to the nucleus, where it displaces the corepressors from LEF-1 and TCF-1, resulting in the formation of a transcription complex, which activates the expression of Wnt target genes (4–7). Wnt signaling is pivotal to numerous aspects of embryonic development and remains essential in some self-renewing tissues throughout life. In hematopoiesis, the canonical Wnt signaling pathway controls the maintenance and self-renewal of hematopoietic stem cells (3, 8, 9), regulates B cell development in fetal liver and neonatal bone marrow (10, 11), and is critically involved in early T cell development in the thymus (12–15). Moreover, components of the Wnt pathway were shown to be expressed in mature, activated T cells, including CD4⁺ T helper (Th)3 cells (16–18). Several recent studies indicate that LEF-1 and TCF-1 are central in Th2 cell development. LEF-1, introduced into developing Th2 cells, was shown to suppress the transcription of Th2-specific cytokines by interacting with GATA3 (GATA-binding protein 3), a key factor for Th2 differentiation. The observed inhibition of cytokines is thought to be a consequence of LEF-1/GATA3 interactions, resulting in reduced binding of GATA3 to the cytokine gene promoters (19). Recently, we identified a high affinity LEF-1-binding site in the negative regulatory element of the IL4 promoter and showed that LEF-1 binds to this element with significantly higher affinity than does TCF-1. Silencing of LEF-1 results in an increase of IL-4 mRNA expression induced in response to stimulation by phorbol 12-myristate 13-acetate/ionomycin, indicating that LEF-1 contributes to the negative regulation of the IL4 gene through transcriptional repression of the IL4 locus (18). Although these studies suggest that LEF-1 is involved in the negative regulation of Th2-specific cytokine production, a very recent study demonstrates that TCF-1 and its co-factor β-catenin promote the differentiation of TCR-activated CD4⁺ T cells into Th2 cells by inducing early GATA3 expression (20). This indicates that LEF-1 and TCF-1 contribute to Th2 cell development in rather different ways.

In the present study, we show that the main Th2 cytokine IL-4 is a potent suppressor of TCF-1 in naive human CD4⁺ T cells. Analyses of TCF-1 protein and mRNA levels revealed that IL-4 signaling preferentially targets the short TCF-1 isoforms, which are constitutive transcriptional repressors. To investigate the molecular mechanisms underlying the IL-4-mediated suppression of TCF-1, we analyzed signaling pathways downstream of the IL-4 receptor and found STAT6 to be crucially involved in the down-regulation of TCF-1.

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†1 The abbreviations used are: Th, T helper; qRT-PCR, quantitative RT-PCR; H3K4, histone 3 Lys4; H3K4me1 and H3K4me3, histone 3 Lys4 single and triple methylation, respectively; TCR, T cell receptor.

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EXPERIMENTAL PROCEDURES

Isolation of Human Peripheral Blood Mononuclear Cells and Preparation of Naive and Memory CD4+ T Cells—All studies involving human cells were conducted in accordance with the guidelines of the World Medical Association’s Declaration of Helsinki. Human peripheral blood mononuclear cells were isolated fromuffy coats of healthy donors by means of Ficoll-Paque Plus® (Amersham Biosciences) density gradient centrifugation and washed twice with PBS (PAA, Pasching, Austria). Naive and memory CD4+ T cells were purified from the prepared peripheral blood mononuclear cells by using the human naive CD4+ T cell isolation kit or the human memory CD4+ T cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer’s protocols. Cells were cultured in x-vivo 15 medium (BioWhittaker, Lonza, Cologne, Germany), supplemented with 5% heat-inactivated human serum AB (BioWhittaker), 2 mM l-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (all purchased from PAA) in plastic tissue culture dishes at 37 °C in a humidified atmosphere containing 5% CO2. T cells were stimulated with plate-bound anti-CD3 at a coating concentration of 10 µg/ml (clone OKT3, eBioscience, Vienna, Austria) and 1 µg/ml soluble αCD28 (BD Pharmingen, Schwachat, Austria). Recombinant human IL-12 (25 ng/ml) (Immunotools, Friesoythe, Germany) and 1 µg/ml recombinant mouse IL-4 (BioWhittaker) were added to cultured cells. Cell isolation was performed by 5% heat-inactivated human serum AB (BioWhittaker), 2 mM l-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (all purchased from PAA) in plastic tissue culture dishes at 37 °C in a humidified atmosphere containing 5% CO2. T cells were stimulated with plate-bound αCD3 at a coating concentration of 10 µg/ml (clone OKT3, eBioscience, Vienna, Austria) and 1 µg/ml soluble αCD28 (BD Pharmingen, Schwachat, Austria). Recombinant human IL-12 (25 ng/ml) (Immunotools, Friesoythe, Germany) and 1 µg/ml recombinant mouse IL-4 (BioWhittaker) were added to cultured cells. Cell isolation was performed by 5% heat-inactivated human serum AB (BioWhittaker), 2 mM l-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (all purchased from PAA) in plastic tissue culture dishes at 37 °C in a humidified atmosphere containing 5% CO2. SDS-PAGE and Immunoblotting—Naive CD4+ T cells were stimulated with IL-4 for the indicated times or left untreated. Cells were harvested by centrifugation, lysed in 2x Laemmli sample buffer (Bio-Rad), and frozen at −75 °C. After thawing, lysates were denatured by a 7-min incubation at 95 °C and afterward centrifuged to remove the cell debris. Protein lysates were separated on a precast NuPAGE 12% or a 4–12% gradient gel (Invitrogen) and blotted onto nitrocellulose membranes in a Trans-blot semidyblotting chamber (both from Bio-Rad). The membrane was blocked by incubation in Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat dry milk for 1 h. The primary antibodies and HRP-linked secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA) and used according to the manufacturer’s instructions. Detection was carried out using Supersignal enhanced chemiluminescence substrate (West Pico, Pierce). For stripping, the membrane was incubated in 50 mM Tris-HCl, pH 6.8, 2% SDS, 10 mM β-mercaptoethanol for 20 min at 50 °C.

RNA Isolation and Quantitative Real-time PCR—Total RNA from cells was isolated using the TRIZol reagent (Invitrogen) and reverse transcribed with RevertAid H Minus M-MuLV reverse transcriptase (MBI Fermentas, St. Leon-Roth, Germany) according to the manufacturer’s instructions. Quantitative real-time PCR was carried out on a Rotorgene 3000 (Corbett Research) using the iQ SYBR Green Supermix (Bio-Rad) and the primers listed below. The transcript for the large ribosomal protein P0 (RPL P0) was used as reference. The specificity of the PCRs was checked by recording a melting curve for the PCR products. Relative mRNA expression levels were calculated using the formula $x = 2^{-ΔCt}$, where $Ct$ represents the threshold cycle of a given gene and $ΔCt$ signifies the difference between the Ct values of the gene in question and the Ct value of the reference gene RPL P0. Induction ratios were calculated using the formula $x = 2^{-ΔΔCt}$, $ΔΔCt$ represents the difference between the $ΔCt$ values of induced and uninduced samples.

Primer sequences are as follows: human TCF-1 sense (5’-CGGGACACGGACCCATTCAACTAGTACAGGAGG-3’) and antisense (5’-CCACCTGCTCGCGGCTGCCAAGAT-3’), human LEF-1 sense (5’-CGACGGCAAGAGGAACACTGACATGC-3’) and antisense (5’-CAGGCAAGAGGAACACTGACATGC-3’), human β-catenin sense (5’-CACTTGATCTGGATCTCGTCACTGCCTCTGGATGG-3’) and antisense (5’-GTCTAATCCATCTTGTGAATCTGGTTGG-3’), mouse TCF-1 sense (5’-AGCCCCCCCCACAGCACTCCTCGAGAGA-3’) and antisense (5’-CAGGTTCCAGGAGTTGTCGACCC-3’), mouse LEF-1 sense (5’-AGCCAGAGGCCAGCCAGCCAG-3’) and antisense (5’-GGGCCTTTGAGTAAAGGAGACACA-3’), human RPL P0 sense (5’-GGCACCATTGAAATCCTCAGTGATGTG-3’) and antisense (5’-TTGGACGACACCCTCCTCAGGAAGG-3’), mouse RPL P0 sense (5’-TGACACTCTCTCTTTCTGAGGTTG-3’) and antisense (5’-AATGCGATGTGATGACCAGGAGG-3’), human TCF-1 p30 sense (5’-GTTGACTGACTAATCCGCCGCCCT-3’) and antisense (5’-AAATTGTTGATGAGAGGAGGTTGGGGGAC-3’), human CCL17 sense (5’-CCAGGGATGCCATGTTTTTGTAACGTGTC-3’) and antisense (5’-CTCACTGTGCTCTTCTTCCTCGACTGAA-3’).
Transfection protocols were performed according to the manufacturer’s instructions. Best transfection efficiency was achieved using $5 \times 10^6$ cells and 100 pmol of siRNA per cuvette and transfection program T-23. Four hours post-transfection, the medium was changed to medium containing 100 units/ml IL-2 (ImmunoTools, Friesoythe, Germany). 72 h post-transfection, cells were incubated for 24 h in the presence or absence of IL-4.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays (EMSAs)—Nuclear extracts from IL-4-induced or non-induced CD4$^+$ T cells were prepared according to the method of Andrews and Faller (22). EMSAs were carried out as described previously (23, 24). Double-stranded oligonucleotide probes corresponding to the sequences −2817 to −2774, −428 to −389, +1673 to +1701, and +708 to +7137, and +20993 to +21022 were used. For supershifting of bands, 200 ng of antibodies (αSTAT6 M20x and αSTAT5 N20x, Santa Cruz Biotechnology, Heidelberg, Germany) was added.

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IL-4 Enhances Suppression of LEF-1 and TCF-1 Expression in TCR-stimulated Naive CD4$^+$ T Cells—Upon antigen challenge, naive CD4$^+$ T cells differentiate into distinct subsets of Th cells. The cytokine milieu in which TCR stimulation takes place is essential to the differentiation of CD4$^+$ cells and determination of Th cell functions. In a recent study, we investigated LEF-1 and TCF-1 expression in a total pool of CD3$^+$ T cells. We found down-regulation of LEF-1 and TCF-1 upon TCR stimulation, which was even more pronounced upon additional IL-4 treatment (18). Because IL-4 is the main cytokine driving the differentiation of Th2 cells, we analyzed LEF-1 and TCF-1 expression in the course of Th1/Th2 differentiation. Naive human CD4$^+$ T cells were isolated and cultured under Th1 (αCD3/αCD28, IL-12) or Th2 (αCD3/αCD28, IL-4) polarizing conditions. After 48 h, mRNA was isolated, and LEF-1 and TCF-1 mRNA expression was determined by qRT-PCR analysis. In line with our previous data, TCF-1 and LEF1 were down-regulated upon TCR activation. Similar to the observations from Yu et al. (20), who demonstrated reduced TCF-1 expression in Th1-skewed mouse CD4$^+$ T cells compared with undifferentiated cells, we found that the addition of the Th1-polarizing cytokine IL-12 resulted in decreased LEF-1 and TCF-1 expression. Nonetheless, the strongest effect was observed when naive T cells were cultured under Th2-polarizing conditions (Fig. 1).

IL-4-induced Suppression of TCF-1 and LEF-1 Is Restricted to Naive CD4$^+$ T Cells—To study the effects of Th1- and Th2-polarizing cytokines in the absence of TCR ligation, naive and memory CD4$^+$ T cells were isolated separately and treated with IL-4 or IL-12 for defined time periods. LEF-1 and TCF-1 mRNA expression was detected by qRT-PCR. Although TCF-1 levels increased in uninduced and IL-12-treated cells over time, the addition of IL-4 resulted in significant down-regulation of TCF-1 mRNA. The down-regulation was rapid (2 h) and persisted for up to 48 h. Similar but time-delayed effects were observed for LEF-1 (Fig. 2, left). In con-
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Contrast to the results for naive CD4+ T cells, neither of the culturing conditions induced changes in the mRNA expression of TCF-1 and LEF-1 in memory CD4+ T cells (Fig. 2, right).

Low IL-4 Concentrations Are Sufficient to Suppress LEF-1 and TCF-1 Expression—To analyze the IL-4-mediated effects in more detail, a concentration kinetics experiment was carried out. mRNA expression of LEF-1, TCF-1, β-catenin, and the IL-4-inducible gene CCL17 (27) was analyzed by means of qRT-PCR. Although β-catenin remained stable, LEF-1 and, to a greater extent, TCF-1 were down-regulated by IL-4 in a concentration-dependent way. Interestingly, very low concentrations of IL-4 (≤50 pg/ml) produced a significant suppression on both LEF-1 and TCF-1, and concentrations of ≤500 pg/ml had a full inhibitory effect. In contrast, up-regulation of CCL17 was not observed at IL-4 concentrations below 500 pg/ml, and its expression increased with rising concentrations of IL-4 up to 50 ng/ml (Fig. 3). Because these results are in line with earlier observations on the expression of IL-4-inducible genes (24, 27), we conclude that TCF-1 and LEF-1 are far more sensitive to IL-4 than are other genes known to be activated by IL-4.

Reduction of TCF-1 Expression Is Restricted to the Short, Inhibitory Isoforms—So far, our data show that IL-4 alone exhibits a potent inhibitory effect on TCF-1 expression. For detailed analyses of TCF-1 expression and data interpretation, it has to be considered that at least eight isoforms of TCF-1 have been described. These result from alternative promoter usage, the presence of alternative exons, and the usage of three different C-terminal splice acceptor sites (25, 28) and can be roughly divided into two groups. The long isoforms (42–60 kDa) contain a β-catenin interaction domain, which is essential for transcriptional activation (25, 29), a Groucho protein-binding domain known to mediate transcriptional repression (4), and a C-terminal DNA-binding domain. In contrast, the short TCF-1 isoforms (25–40 kDa) lack the β-catenin interaction motif but contain Groucho- and DNA-binding sites (Fig. 4A). Therefore, the shorter TCF-1 isoforms act as persistent transcriptional repressors. Transcripts of short and long TCF-1 isoforms can be distinguished by the presence of exon 1, which is exclusively found in the short isoforms. To detect the short TCF-1 isoforms by means of qRT-PCR, a forward primer binding to exon 1 was designed. Although expression of the short isoforms was dramatically inhibited by IL-4 (reduction of 92%), the effects observed on total TCF-1 expression were less pronounced, indicating that IL-4 preferentially down-regulates the expression of short TCF-1 isoforms (Fig. 4B).
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Next we investigated TCF-1 protein expression by Western blotting. Analysis of TCF-1 protein revealed no inhibition of the long TCF-1 isoforms upon IL-4 stimulation. In contrast, shorter isoforms were significantly down-regulated after 24–48 h of IL-4 treatment. In line with our mRNA expression results, these data indicate a specific effect of IL-4 on the expression of short TCF-1 isoforms. TCF-1 isoform expression in a Jurkat cell lysate was detected as a control. To control for efficient IL-4 stimulation and equal loading, phosphorylated STAT6 and total STAT6 were detected. A Jurkat cell lysate was loaded as control for TCF-1 isoform expression.

STAT6 Binds to Specific Binding Motifs within the Human TCF1 Locus—Engagement of the IL-4 receptor results in the activation of Janus kinases (Jaks), which subsequently phosphorylate tyrosine residues on IL-4Rα, thus providing docking sites for STAT6. Once bound to the receptor, STAT6 monomers become phosphorylated, dimerize, and translocate to the nucleus, where the active STAT6 dimers bind to cognate-responsive DNA elements and initiate IL-4-mediated transcription (30). To date, more than 35 genes regulated by STAT6 have been described (30). Although the vast majority of these genes are positively regulated, IL-4-mediated STAT6 activation was also described to have suppressive effects on gene expression (31, 32). Analysis of the TCF-1 locus revealed the presence of four putative STAT6-binding sites characterized by the sequence TTCN₄GAA (Fig. 5A). These motifs are located at positions −2804 (site 1), −412 (site 2), +1678 (site 3), and +7122 (site 4) relative to the translational start site in exon 1a described by van de Wetering et al. (25). Besides the putative STAT6-binding sites, an additional STAT consensus motif was identified at position +21,001 (site 5). This site is not a classical STAT6 consensus binding site because it carries only three nucleotides dividing the palindromic half-sites TTC/GAA and therefore rather resembles the consensus motif for STAT1, STAT3, or STAT5. Because it was reported that IL-4 can also activate STAT5 in lymphocytes (33, 34), we included this site in our study. The interaction of STATs with putative STAT-binding sequences within the TCF-1 locus was assessed by EMSAs. Nuclear extracts prepared from untreated and IL-4-treated naive CD4⁺ T cells were incubated with double-stranded oligonucleotide probes harboring the sequences −2804/−2815 (site 1), −412/−423 (site 2), +1678/+1687 (site 3), +7122/+7131 (site 4), and +21001/+21009 (site 5), and nucleoprotein complexes were resolved in native polyacrylamide gels. Incubation of nuclear extracts from untreated or IL-4-induced cells with any of the four oligonucleotides harboring a classical STAT6-binding sequence resulted in the formation of an IL-4-specific nucleoprotein complex (Fig. 5B). In contrast, no IL-4-induced complex formation was observed using the TTCN₄GAA motif at position +21001 (site 5). Mutation of the STAT6 consensus site TTCN₄GAA to TATN₄GAA was shown to abrogate binding of STAT6 in previous studies (24, 35–37). By introducing this mutation into the STAT6 consensus motifs within the oligonucleotides from the human TCF1 locus, IL-4-induced complex formation was inhibited (Fig. 5C). For competition assays, a 50-fold molar excess of unlabeled wild-type oligonucleotide was added to the binding reaction. This resulted in the loss of the IL-4-induced formation of the radiolabeled nucleoprotein complexes. In contrast, the addition of mutated oligonucleotide had no effect. The addition of anti-STAT6 antibodies (M20) prior to the addition of radiolabeled probes specifically reduced the formation of the IL-4-induced formation of the radiolabeled nucleoprotein complexes and partly led to the formation of a supershifted complex (sites 1, 3, and 4). An antibody directed against STAT5 (N20), which was used as a control antibody, did not affect the IL-4-induced complex (Fig. 5C). These data suggest that IL-4 stimulates the interaction of STAT6 and specific DNA motifs within the human TCF1 locus.

For in vivo binding of transcription factors to genomic DNA, the chromatin has to be in an open conformation. Methylation of histone 3 on lysine residue 4 (H3K4) is a known marker for chromatin that is amenable to transcription factor binding (26). Wei et al. (38) recently showed that functional STAT6-binding sites are often associated with tri-
ple-methylated H3K4 (H3K4me3) islands. Barski et al. (26) performed chromatin immunoprecipitation/sequencing-based profiling of histone modifications in human CD4\(^+\) T cells. Alignment of these data with the human TCF1 locus shows the presence of triple and single methylated H3K4 islands, which are co-localized with STAT consensus motifs (Fig. 6A). To evaluate the capacity of each motif to bind STAT6 in living cells, we performed chromatin immunoprecipitation with STAT6-specific antibodies. The enrichment of specifically precipitated DNA containing the STAT6 binding sites was analyzed by qRT-PCR using site-specific primer pairs. Control immunoprecipitations with normal rabbit IgG serum resulted in equal amplification of IL-4 and uninduced samples for all four STAT6-binding sites. In contrast, precipitation of chromatin from IL-4-induced cells with anti-STAT6 resulted in enrichment of the STAT6-binding motifs 2, 3, and 4. For motif 1, only slight differences between unstimulated and IL-4-induced cells were detected (Fig. 6A). These findings indicate that sites 2, 3, and 4 are possibly more relevant for IL-4-mediated TCF-1 suppression than site 1. To control for appropriate IL-4 effects, TCF-1 expression and STAT6 activation were analyzed (Fig. 6B).

FIGURE 5. STAT6 binds to specific DNA motifs within the human TCF1 gene. A, schematic representation of the human TCF1 genomic locus as described by van de Wetering et al. (29). Positions of the five putative STAT-binding motifs are indicated relative to the translational start site. B, EMSAs using radiolabeled probes harboring the sequences -2804/-2815 (site 1), -412/-423 (site 2), +1678/+1687 (site 3), +7122/+7131 (site 4), and +21001/+21009 (site 5) were performed. Nuclear extracts prepared from untreated and IL-4-treated naive CD4\(^+\) T cells were incubated with double-stranded oligonucleotide, and nucleoprotein complexes were resolved in native polyacrylamide gels. C, EMSAs using radiolabeled probes comprising wild-type STAT6 motifs (WT) or mutated motifs (mut) were performed. For competition assays, a 50-fold molar excess of unlabeled wild-type or mutated oligonucleotide was added to the binding reaction. In supershift experiments, nuclear extracts were preincubated with an antibody directed against STAT6 (M20) or a control antibody (N20, directed against STAT5).
STAT6 Is Critically Involved in IL-4-induced Suppression of Inhibitory TCF-1 Isoforms—To further investigate the role of STAT6 in the IL-4-induced inhibition of the short TCF-1 isoforms, we analyzed TCF-1 expression in STAT6-deficient, naive human CD4 \(^+\) T cells. Silencing of STAT6 was performed via transfection of naive human CD4 \(^+\) T cells with siRNA targeting STAT6. Decreased levels of STAT6 protein but unaltered STAT1 levels were detected 96 h post-transfection by Western blotting (Fig. 7A). Three days post-transfection, cells were incubated overnight in the presence or absence of IL-4. Real-time PCR analyses showed that stimulation with IL-4 inhibited the expression of TCF-1, especially the short isoforms, in control cells, whereas silencing of STAT6 levels were detected 96 h post-transfection by Western blotting (Fig. 7A). Three days post-transfection, cells were incubated overnight in the presence or absence of IL-4. Real-time PCR analyses showed that stimulation with IL-4 inhibited the expression of TCF-1, especially the short isoforms, in control cells, whereas silencing of STAT6 showed diminished TCF-1 mRNA expression after IL-4 stimulation, whereas in T cells derived from STAT6 \(^{-/-}\) mice, effects on TCF-1 expression were clearly less pronounced and occurred in a time-delayed fashion (Fig. 6C, top). Similar, but less prominent, effects were observed for LEF-1 expression (Fig. 6C, bottom). These data show that STAT6 mediates the IL-4-induced down-regulation of the transcription factors LEF-1 and TCF-1, in particular the short, suppressive TCF-1 isoforms, in naive CD4 \(^+\) T cells.

**DISCUSSION**

Although the role of Wnt pathway-associated transcription factors in early thymocyte development is well documented, the function of LEF-1 and TCF-1 at later stages of T cell development remained ambiguous for a long time. We and others recently showed that LEF-1 and TCF-1 play a crucial role in the regulation of Th2 cell development. We reported that LEF-1 acts as a transcriptional repressor of IL-4 by binding to a negative response element within the IL4 locus. Activation of cells through TCR engagement down-regulates LEF-1 and thereby releases IL4 from its transcriptional repressor. Once expressed, IL-4 enhances the inhibition of LEF-1 and thus...
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Further amplifies IL-4 expression via a positive feedback loop (18). Although LEF-1 regulates IL-4 via a negative feedback loop, TCF-1 was reported to promote Th2 differentiation by activating the Th2-specific transcription factor GATA3 (20). These opposing effects of LEF-1 and TCF-1 in mature T cells are of particular interest because studies analyzing the functions of LEF-1 and TCF-1 during early T cell development revealed a strong functional redundancy between the two transcription factors (39). Our data show that TCF-1 is strongly down-regulated in CD4⁺ T cells cultured under Th cell-polarizing conditions (CD3/CD28/polarizing cytokine). TCR-dependent signals are a common prerequisite for Th cell differentiation. However, the cytokine milieu is the most important factor determining the fate of Th cell differentiation. When we compared the effects of the Th1-polarizing cytokine IL-12 with the Th2-polarizing cytokine IL-4 in the absence of TCR stimulation, we observed a significant suppression of TCF-1 in IL-4-treated, but not in IL-12-treated, naive CD4⁺ T cells. This might be explained by the observation that resting naive T cells do not express the IL-12 receptor subunit β2, which is essential for IL-12 signaling and up-regulated upon T cell activation (40). Thus, IL-12 might also have an inhibitory effect on TCF-1 mRNA expression, but, unlike IL-4, cannot exert it in the absence of TCR stimulation.

Whereas IL-4 down-regulates TCF-1 expression, we observed increasing TCF-1 mRNA levels in untreated and IL-12-stimulated cells within the first 6 h of in vitro culture. This observation may be due to the fact that removing T cells from their in vivo environment leads to the loss of certain stimuli, such as IL-7. Yu et al. (41) demonstrated that IL-7 is involved in the inhibition of TCF-1 in mature peripheral T cells. Because IL-7 is not produced by T cells (42–44), loss of IL-7 signals in the course of in vitro culturing may therefore result in increasing TCF-1 expression. When we investigated TCF-1 expression in memory CD4⁺ cells compared with naive CD4⁺, we found that the overall TCF-1 expression in memory cells was rather low, and none of the tested cytokines showed any effect. This is in line with several other studies showing that TCF-1 is down-regulated upon T cell activation (16, 18). Moreover, this finding indicates that TCF-1 plays a crucial role especially during early Th cell development.

STAT proteins are a family of transcription factors involved in signal transduction induced by various cytokines. STAT6 is the main transcription factor mediating IL-4-induced signaling, as demonstrated by the greatly diminished functions of IL-4 in STAT6-deficient mice (21, 45). However, STAT5 was shown to be activated by IL-4 in lymphocytes too (33, 34). This activation seems to be independent of tyrosine phosphorylation of IL-4Rα but may rather be mediated by the direct interaction of STAT5 and Janus kinases (46). Despite the presence of a putative STAT5-binding site identified in the TCF1 locus, we did not observe IL-4-induced binding of STAT5 to TCF1 gene sequences. In contrast, all four identified STAT6 motifs seem to be capable of binding STAT6, as shown by gel shift assays, indicating that STAT6 is the crucial factor involved in TCF-1 suppression. This assumption was confirmed when we performed knockdown experiments targeting STAT6 in naive human CD4⁺ T cells. IL-4 inhibited TCF-1, in particular the short isoforms, in control cells,
whereas no significant effect of IL-4 was observed in STAT6-deficient cells. Similar results were obtained when we analyzed naive T cells from STAT6−/− mice compared with WT mice.

To validate the binding capacity of each STAT6 motif identified in the human TCF1 locus, ChIP assays were carried out. Analyses of histone modifications along the TCF1 locus revealed that the chromatin appears to be accessible for transcription factor binding at all four binding sites because we found an island of H3K4me1 co-localized with site 1 and H3K4me3 modifications surrounding sites 2, 3, and 4. ChIPs with STAT6-specific antibodies indicate that sites 2–4 seem to be more relevant for IL-4-mediated TCF-1 suppression than site 1. This is in agreement with the study of Wei et al. (38), who showed that STAT6-binding sites and triple methylated H3K4 (H3K4me3) islands often overlap. Noteworthy, analyses of histone modifications along the TCF1 locus re-analyzed naive T cells from STAT6−/− mice compared with WT mice. Since the chromatin appears to be accessible for transcription factor binding at all four binding sites, we found an island of H3K4me1 co-localized with site 1 and H3K4me3 modifications surrounding sites 2, 3, and 4. ChIPs with STAT6-specific antibodies indicate that sites 2–4 seem to be more relevant for IL-4-mediated TCF-1 suppression than site 1. This is in agreement with the study of Wei et al. (38), who showed that STAT6-binding sites and triple methylated H3K4 (H3K4me3) islands often overlap. Noteworthy, binding of STAT6 to specific motifs within the TCF1 locus is transient. The highest occupancies of STAT6 binding motifs were observed after 4 h of IL-4 stimulation, whereas no differences between IL-4-stimulated and untreated samples were detectable after 20 h of IL-4 treatment (data not shown).

Thus, we propose that STAT6 is particularly involved in early IL-4-mediated effects on TCF-1. This assumption is substantiated by the fact that STAT6 deficiency does not entirely rescue TCF-1 from IL-4-mediated suppression as shown in human and mouse naïve T cells.

For a long time, IL-4 and the IL-4-induced STAT6 signaling pathway were regarded as principal factors driving Th2 polarization. Activated STAT6 induces the transcription of GATA3, which is known to be a master regulator of Th2 differentiation. However, several studies showed that IL-4/STAT6 signaling can be bypassed to generate Th2 cells by other pathways, including Notch (47, 48) and IL-2/STAT5 signaling (49, 50). The fact that Th2 differentiation still occurs in STAT6-deficient mice (51) further indicates that STAT6-independent mechanisms exist that may be sufficient to activate GATA3 transcription. A recent study provides clear evidence that early after the activation of CD4+ T cells, TCF-1 induces GATA3 expression from GATA3-1b transcripts in an IL-4- and STAT6-independent way (20). GATA3 expression is driven by two different promoters, both of which are used to transcribe GATA3 in T cells. Activation of the two distinct promoters results in mRNA transcripts that can be distinguished by the presence of promoter-specific sequences unique to exon 1a (GATA3-1a) and exon 1b (GATA3-1b) (52). Whereas GATA3-1a expression is increased after 2–4 days of stimulation and clearly depends on STAT6, GATA3-1b seems to be more active during the early phase of Th2 differentiation and is activated even in the absence of STAT6 (20, 53). The fact that TCF-1 promotes the activation of GATA3-1b transcripts indicates that TCF-1 and STAT6 act through different mechanisms in the regulation of early and delayed GATA3 expression.

In the present study, we demonstrate IL-4/STAT6-dependent suppression of short TCF-1 isoforms in naive CD4+ T cells, which results in the exclusive expression of long TCF-1 isoforms. Differential regulation of the isoforms, as shown here, is of particular interest because long and short TCF-1 isoforms have distinct functions during Th2 cell development. Yu et al. (20) reported that TCF-1 induces GATA3-1b transcription in T cells, resulting in a Th2 phenotype characterized by enhanced IL-4 production. The same study demonstrated that overexpression of a mutated TCF-1 form resembling the naturally occurring short TCF-1 isoforms showed the opposite effect and resulted in decreased IL-4 production (20), indicating that the short isoforms act as inhibitors of TCF-1-mediated Th2 differentiation. Therefore, we suggest that specific down-regulation of inhibitory TCF-1 isoforms, as shown in the present study, contributes to early TCF-1-mediated Th2 differentiation.

Based on our findings, we propose an IL-4/STAT6-dependent fine tuning mechanism emerging at the initial phase of Th2 differentiation. At this stage of Th2 cell development, very low amounts of IL-4 are produced. Notably, the suppression of the short, inhibitory TCF-1 isoforms occurs at an IL-4 concentration of less than 0.5 ng/ml, which is probably not sufficient to induce STAT6-driven, GATA3-1a-dependent Th2 differentiation. Although not capable of inducing the STAT6-dependent GATA3-1a transcript, the present IL-4 still is sufficient to contribute to early TCF-1-mediated GATA3-1b transcription by suppressing the inhibitory TCF-1 isoforms. As a consequence, the developing Th2 cells generate large amounts of IL-4, and the classical IL-4/STAT6-dependent pathway allows for the manifestation of the Th2 phenotype. Thus, STAT6 promotes the development of Th2 cells not only through direct interaction with the GATA3 promoter but also through down-regulation of the natural inhibitors of the newly described, TCF-1-dependent, alternative Th2 differentiation pathway.

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