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LEF-1 Negatively Controls Interleukin-4 Expression through a Proximal Promoter Regulatory Element*

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Abstract

Lymphoid enhancer-binding factor 1 (LEF-1) and T cell factor (TCF-1) are downstream effectors of the Wnt signaling pathway and are involved in the regulation of T cell development in the thymus. LEF-1 and TCF-1 are also expressed in mature peripheral primary T cells, but their expression is down-regulated following T cell activation. Although the decisive roles of LEF-1 and TCF-1 in the early stages of T cell development are well documented, the functions of these factors in mature peripheral T cells are largely unknown. Recently, LEF-1 was shown to suppress Th2 cytokines interleukin-4 (IL-4), -5, and -13 expression from the developing Th2 cells that overexpress LEF-1 through retrovirus gene transduction. In this study, we further investigated the expression and functions of LEF-1 and TCF-1 in peripheral $CD4^+$ T cells and revealed that LEF-1 is dominantly expressed in Th1 but not in Th2 cells. We identified a high affinity LEF-1-binding site in the negative regulatory element of the IL-4 promoter. Knockdown LEF-1 expression by LEF-1-specific small interfering RNA resulted in an increase in the IL-4 mRNA expression. This study further confirms a negative regulatory role of LEF-1 in mature peripheral T cells. Furthermore, we found that IL-4 stimulation possesses a negative effect on the expressions of LEF-1 and TCF-1 in primary T cells, suggesting a positive feedback effect of IL-4 on *IL4* gene expression.

The development and differentiation of T cells is a spatially and temporally diverse process. Although input from the T cell receptor (TCR)[2] affects T cells at most differentiation stages, later stages of the maturation process including polarization into T helper 1 (Th1) and Th2 subsets depend primarily on the cytokine milieu in the periphery (1). Instead, the earlier development takes place in the thymus and is influenced by developmental pathways like the Wnt cascade (2, 3).

The Wnt signaling pathway is critically involved in various biological phenomena including determination of cell fate, proliferation of progenitor cells, establishment of polarity, and

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gene expression (3, 4). Aberrant activation or disruption of the Wnt signaling pathway have been implicated in developmental defects in bone mass, teeth, Tetra-amelia, and also in many types of cancers (5–7). The canonical Wnt cascade is initiated by binding of Wnt ligands to their cognate receptor complex, a member of the Frizzled family. This leads to destabilization of the β-catenin degradation complex composed of adenomatous polyposis coli, Axin, and glycogen-synthase kinase 3-β. Once this complex disassembles, the cytoplasmic levels of β-catenin rise. In the absence of Wnt signaling, glycogen-synthase kinase 3-β phosphorylates β-catenin, which ultimately leads to its proteasomal cleavage. The rise of the cytoplasmic levels of β-catenin allows its nuclear accumulation, where it interacts with members of the lymphoid enhancer-binding factor (LEF) and T cell factor (TCF) family of transcription factors and thereby activates target genes (5). Although LEF-1/TCF-1 can directly bind to DNA, they are incapable of independently activating gene transcription. Rather, they function as transcription repressors by complexing with members of the Groucho-related gene family (8–10). In addition, both LEF-1 and TCF-1 possess isoforms that may act in a dominant negative way (11).

Wnt signaling is strongly associated with normal hematopoiesis. In particular, LEF-1 and TCF-1 have been shown to influence several checkpoints of developing T cells in thymus (2, 5, 12). LEF-1 is expressed in most cells of the T cell lineage and was originally identified as a lymphoid-specific DNA-binding protein that recognizes a 5′-CTTTGAA motif in the TCRα enhancer (13). Similarly, TCF-1 was identified as a factor binding to the same TCRα enhancer site and represents the first T cell marker expressed in the most immature $CD4^{-}CD8^{-}$ developing T cells in fetal thymus (14–16). TCF-1 knock-out mice displayed impaired T cell development from immature stages on (17–19). Although LEF-$1^{-/-}$ mice were reported to have a normal T cell population (20), TCF-$1^{-/-}$ LEF-$1^{-/-}$ double knock-outs, which are embryonically lethal, did not only show impairment of the $CD4^{-}CD8^{-}$ thymocyte subsets but also a more severe defect in T cell development at the immature $CD4^{-}CD8^{+}$ stage. This suggests a redundant role of these factors (21, 22). The role of Wnt signaling in lymphopoiesis is further evidenced by inducible knock-out of the β-catenin gene, which resulted in impairment of T cell development at the TCR β-chain checkpoint (23).

Although the decisive role the Wnt pathway plays in earlier stages of T cell development is well documented, evidence on Wnt signaling in mature peripheral T cells is scarce. β-Catenin was reported to be expressed at very low or undetectable levels in mature peripheral blood T cells compared with malignant T cells (24). Also, TCF-$1^{-/-}$ mice were described as

[2]The abbreviations used are:

TCR T cell receptor

LEF lymphoid enhancer-binding factor

IL interleukin

TCF T cell factor

siRNA small interfering RNA

Th T helper

PMA phorbol 12-myristate 13-acetate

EMSA electrophoretic mobility shift assay.

fully immunocompetent, suggesting that TCF-1 is essential for maintenance of early thymocyte progenitors but may be dispensable in more mature T cells (18, 25). However, recent studies found that LEF-1 and TCF-1 are expressed in mature naïve T cells, and the expression levels of LEF-1 and TCF-1 are down-regulated after TCR stimulation (26, 27). More recently, LEF-1 was shown to be able to interact with the Th2-specific transcription factor GATA-3, and introduction of LEF-1 into developing Th2 cells resulted in reduction of the Th2 cytokines IL-4, IL-5, and IL-13 productions (27). Therefore, LEF-1 and TCF-1 may be important for silencing transcription in peripheral T cells.

In this study, we further investigated the expression and function of LEF-1 and TCF-1 in $CD4^+$ T cells. We found that LEF-1 is expressed dominantly in Th1 but not in Th2 cells. We identified a high affinity LEF-1-binding site in the proximal promoter region of the Th2-specific cytokine IL-4 and confirmed that LEF-1 negatively controls the *IL4* gene expression. Furthermore, we show that IL-4 stimulation inhibits expressions of LEF-1 and TCF-1 in primary T cells, demonstrating a positive feedback effect of IL-4 on *IL4* gene expression.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture

The cell lines used in this study were the human T cell leukemia cell line Jurkat, the mouse Th2 clone D10, and the mouse Th1 clone 29 (C29) (28). Jurkat and human peripheral T cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum, 50 μg/ml gentamicin (Invitrogen), or 100 units/ml penicillin and 100 units/ml streptomycin, 6 mM HEPES (Invitrogen; 1 M solution), and 2 mM L-glutamine (Invitrogen; 200 mM solution) at 37 °C in a humidified atmosphere containing 5% CO_2. D10 and C29 cells were cultured as above supplemented with 2 units/ml IL-1, 25 units/ml IL-2, and 2 mg/ml Con A (Sigma).

Preparation of Human Peripheral Blood T and $CD4^+$ T Cells

Human peripheral T cells were prepared as described previously (29) and were more than 90% CD3 positive. $CD4^+$ T cells were isolated from the purified T cells by human CD4 MicroBeads (MACS Miltenyi Biotec., Bergisch Gladbach, Germany) according to the manufacturer's instructions. For T cell stimulation, recombinant human IL-4 (a generous gift from Novartis, Vienna, Austria) was used at a concentration of 50 ng/ml. For T cell activation, phorbol 12-myristate 13-acetate (PMA) (20 ng/ml) (Sigma) and ionomycin (2 μM) (Calbiochem) or αCD3/αCD28-coated beads (Invitrogen) at a final concentration of 1×10^6 beads/ml were used.

In Vitro Th1/Th2 Differentiation

The *in vitro* Th1/Th2 differentiation was carried out by the established method (30). Briefly, naïve $CD4^+$ T cells isolated from the mouse spleen and $CD4^+CD62L^+$ naïve cells were purified via MACS and were cultured on plates precoated with α-CD3 (1 μg/ml) and α-CD28 (5 μg/ml). Th1 conditions were established by using IL-12 (3.4 ng/ml), IL-2 (20 units/ml), and α-IL-4 antibody (2 μg/ml) (BD Transduction Laboratories). Th2 conditions were established by using IL-4 (3000 units/ml), IL-2 (20 units/ml), and α-interferon-γ antibody (2 μg/ml) (BD Transduction Laboratories). Forty-eight hours after starting the culture, the cells were replated to fresh medium containing the above polarizing cytokines and anti-cytokine antibodies plus IL-2 (5 units/ml). The cells were cultured further for another 2 days and then washed and stimulated with plate-bound α-CD3 for the indicated times.

Western Blot Analysis

1×10^6 cells were sedimented and lysed for 15 min in ice-cold tysis buffer (15 mM Tris-HCl, pH 7.4, 137 mM NaCl, 10% (w/v) glycerol, 1% (v/v) Triton X-100, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.4 mM Na_3VO_4, 10mM NaF, and complete protease inhibitor mixture; Roche Applied Science). After removing the cell debris by centrifugation at 13,000 rpm for 15 min, equal amounts of proteins were separated on a 12% SDS-PAGE under reducing conditions, blotted onto a nitrocellulose membrane (Amersham Biosciences), and blocked with 5% nonfat dry milk in 0.05% Tween 20 in phosphate-buffered saline. The following antibodies were used: anti-human/mouse LEF-1 polyclonal antibody (N-17; Santa Cruz Biotechnology), anti-LEF-1 monoclonal antibody (AAH50632; Abnova Corporation, Taipei, Taiwan), anti-human TCF-1 7H3 (Upstate Biotechnology), anti-human/mouse TCF-1 (H-118; Santa Cruz Biotechnology), anti-β-catenin (BD Transduction Laboratories), anti-STAT6 M-20 (Santa Cruz Biotechnology), anti-pSTAT6 (Cell Signaling Technology), anti-pIκBα (Cell Signaling Technology), anti-active p38 antibody (Promega, Heidelberg, Germany), anti-p38 (5F11) (Cell Signaling), anti-GATA-3 mAb HG3–31 (Santa Cruz Biotechnology), and anti-YY1 (Santa Cruz Biotechnology). As secondary antibodies, we employed anti-mouse or anti-rabbit horseradish peroxidase conjugates (Bio-Rad). The blots were detected by means of enhanced chemiluminescence (Pierce). Stripping was achieved by incubating the membrane in 62.5 mM Tris HCl, 2% SDS, 100 mM β-mercaptoethanol at 65 °C for 20 min.

Quantitative Real Time PCR

Total RNA was isolated from the cells using either the TRIzol (Invitrogen) or the RNeasy kit (Qiagen) according to the manufacturer's instructions. Quantitative analysis of the IL-4 mRNA expression was carried out as described previously (31). For quantitative analysis of LEF-1, TCF-1, and SOCS-1 mRNA expression, 4 μg of the total RNA was reversely transcribed with RevertAid H Minus Moloney murine leukemia virus reverse transcriptase (MBI Fermentas) following the manufacturer's protocol. The PCR was run on a Rotorgene 2000 (Corbett Research) using the iQ SYBR Green Supermix (Bio-Rad). The primers were designed to amplify targets of 180–220 bp from the 3′-untranslated region of the mRNAs. The gene for large ribosomal protein P20 (RPLP0) was used as reference. Sequences of the primers are listed below. The specificity of the PCRs was checked by recording a melting curve and by sequencing the amplicons on an ABI prism automated sequencing machine. Induction ratios (x) were calculated using the formula $x = 2^{-\Delta\Delta Ct}$, where C_t represents the threshold cycle of a given gene, and ΔC_t represents the difference between the C_t values of the gene in question and the C_t value of the reference gene (large ribosomal protein P0). $\Delta\Delta C_t$ is the difference between the ΔC_t values of the "induced" samples and the ΔC_t of the corresponding "noninduced" sample. The mean induction ratios of all replicate analyses were calculated. The sequences of the primers are as follows: RPLP0, forward, 5′-GGCACCATTGAAATCCTGAGTGATGTG-3′, and reverse, 5′-TTGCGGACACCCTCCAGGAAGC-3′; hSOCS-1, forward, 5′-TTGGAGGGAGCGGATGGGTGTAG-3′, and reverse, 5′-AGAGGTAGGAGGTGCGAGTTCAGGTC-3′; hLEF-1, forward, 5′-CGACGCCAAAGGAACACTGACATC-3′, and reverse, 5′-GCACGCAGATATGGGGGGAGAAA-3′; hTCF-1, forward, 5′-CGGGACAGAGGACCATTACAACTAGATCAAGGAG-3′, and reverse, 5′-CCACCTGCCTCGGCCTGCCAAAGT-3′; mLEF-1, forward, 5′-AGCCAAGGCAGCGACCCCAGG-3′, and reverse, 5′-CGGCGCTTGCAGTAGACGACAGA-3′; and mTCF-1, forward, 5′-CCCCCCACAGCACCCTCCAGAATC-3′, and reverse, 5′-CCAGGTTCAGGGAGTTGTGCAGCC-3′.

Recombinant TCF-1 and β-Catenin Proteins

TCF-1 and β-catenin bacterial expressing plasmids were constructed by cloning the full length of human TCF-1 and β-catenin cDNAs (generated from Jurkat T cells) into the bacterial expression vector pGEX5X1. The following PCR primers were used to generate the restriction enzyme sites EcoRI/XhoI and BamHI for cloning TCF-1 and β-catenin cDNAs, respectively: for TCF-1, 5′-CCGGCCGAATTCATGTACAAAGAGACCGTCTAC-3′ and 5′-GGCCGGCTCGAGTCAGGGGTAGGCTCCTG-3′; for β-catenin, 5′-CCGGCCGGATCCGGATGGCTACTCAAGCTGATTTG-3′ and 5′-CCTTACAGGTCAGTATCAAACCA-3′. The plasmids were transformed into bacterial strain DH5α and cultured at 37 °C at an optical density of up to 0.6 in LB medium containing 50 μg/ml ampicillin. The bacterial were cooled down to 20 °C for 30 min and were induced by isopropyl β-D-thiogalactopyranoside at a final concentration of 0.1 mM for 1 h. The bacterial were collected by centrifugation at 7000 rpm for 10 min and were suspended in 150 mM NaCl, 16 mM Na_2HPO_4, and 4 mM NaH_2PO_4 containing protease inhibitors. The bacteria were lysed by sonification and than centrifuged at 8000 rpm for 15 min. The recombinant proteins were purified from the supernatant using glutathione-Sepharose 4B (Amersham Biosciences) according to the manufacturer's instructions. The purified recombinant proteins were checked by SDS-electrophoresis and Western blot.

Nuclear Extract, EMSA, and Methylation Interference Analysis

The preparation of nuclear extracts, EMSA, and methylation interference analysis were performed as described previously (32). The IL-4 promoter sequence used for EMSA and methylation interference is 5′-TGCTGAAACTTTGTAGTTAATTTTG-3′. The synthetic oligonucleotide of the TCF-1/LEF-1 consensus binding site for EMSA is 5′-TCCCTTTGATCTTACCG-3′. The control oligonucleotide containing the binding site for NF-Y (Eα) is 5′-TATTTTTCTGATTGGTTAAAAGTG-3′.

LEF-1 siRNA

The LEF-1 knockdown experiment was carried out with the SureSilencing™ shRNA plasmid encoding siRNA against LEF-1 (KH02778G/N), and the negative control plasmid was purchased from Biomol GmbH (Hamburg, Germany). The plasmids were transfected by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The positively transfected cells were selected for resistance to neomycin. The efficiency of knocking down LEF-1 was controlled by real time PCR and Western blot analysis.

RESULTS

LEF-1 Is Dominantly Expressed in Th1 but Not in Th2 Cells

During an immune response, $CD4^+$ Th cells undergo differentiation into either Th1 or Th2 effector cells. Because LEF-1 and TCF-1 expression were down-regulated following T cell activation (26, 27), we asked whether LEF-1 and TCF-1 were expressed at all in differentiated effector Th cells. To investigate this question, a Th1 clone (C29) and a Th2 clone (D10) were subjected to the real time PCR analysis. We found that the LEF-1 mRNA was expressed at a significantly higher level in Th1 C29 than in Th2 D10 cells (Fig. 1*A*). In contrast to LEF-1, almost no TCF-1 mRNA could be detected in either Th1 C29 or Th2 D10 cells (Fig. 1*A*). To confirm this observation, nuclear extracts were prepared from the Th1 C29 and Th2 D10 cells, and the expression patterns of LEF-1 and TCF-1 in these cells were examined by Western blot analysis. Interestingly, we found that LEF-1 was expressed in Th1 C29 but not in Th2 D10 cells (Fig. 1*B*). Corresponding to the mRNA expression levels, TCF-1 proteins were expressed at almost undetectable levels in both Th1 and Th2 cells.

Only a band at ~28 kDa in C29 and a band at 24 kDa in D10 were found at positions similar to those of the inhibitory TCF-1 isoforms (Fig. 1*B*, indicated by an *asterisk*). The absence of LEF-1 in D10 cells was not due to unequal loading of the nuclear extracts. As shown, both cell lines express similar levels of the ubiquitous factor YY1 and also a strong expression of the Th2-specific nuclear factor GATA-3 was seen in D10 cells, demonstrating that the absence of LEF-1 was not due to different qualities of the nuclear extracts. To further confirm this observation, naïve $CD4^+$ cells isolated from mouse spleen were subjected to differentiation under either Th1 or Th2 differentiation culture conditions (see "Experimental Procedures"). The newly differentiated Th1 and Th2 cells were analyzed by real time PCR and Western blot analysis. Consistent with the observations from the Th1 C29 and Th2 D10 cell lines, LEF-1 was dominantly expressed in freshly differentiated Th1 cells (Fig. 1, *C* and *D*). TCF-1, on the other hand, was expressed at low levels in both cell types.

The IL-4 Promoter Contains a High Affinity Binding Site for LEF-1

Overexpression of LEF-1 in the developing Th2 cells has been shown to suppress IL-4, -5, and -13 expressions (27). We have previously identified a negative regulatory element in the IL-4 promoter −225 to −201 regions (Fig. 2*A*). Point mutations within this element resulted in increases in the IL-4 promoter activity (32). Interestingly, we found that this negative regulatory element shares DNA sequence homology to the LEF/TCF 5′CTTTG(A/T)(A/T)-binding motif. Because LEF-1 and TCF-1 may function as transcriptional repressors (8–10), we asked whether LEF-1 and TCF-1 interact with this DNA sequence. To investigate this question we carried out a methylation interference analysis to examine the precise DNA-binding sites of nuclear proteins at this region. Because Jurkat T cells express all three Wnt pathway proteins: β-catenin, LEF-1, and TCF-1, nuclear extracts from Jurkat T cells were used for this assay. The experiment showed that the DNA/protein contact sites were exactly located within the LEF/TCF homologous region (Fig. 2, *B* and *C*). To further investigate whether the DNA contact sites observed by the methylation interference analysis were caused by binding of LEF-1 and TCF-1, a probe containing the IL-4 promoter nucleotide −225 to −201 (probe IL4-Lef) and nuclear extracts from Jurkat T cells were used in EMSA. A DNA-protein complex formed by the IL4-Lef probe was shown to be supershifted by the α-LEF-1 antibody, demonstrating that LEF-1 was involved in binding to the IL-4 TCF/LEF homologous sequence (Fig. 3*A*). Although Jurkat T cells express high amounts of TCF-1 and β-catenin (Fig. 1*B*), surprisingly, no supershifts were seen using the antibodies against TCF-1 and β-catenin (Fig. 3*A*). To investigate whether the IL-4 −225/−201 promoter sequence interacts with TCF-1 and β-catenin at all, recombinant TCF-1 (r-TCF-1) and β-catenin (r-β-catenin) proteins were generated by a bacterial expression system and were used in EMSA. For a positive control, a DNA probe containing the consensus DNA-binding sequence for TCF-1/LEF-1 (probe CS-T/L) was used in parallel. EMSA showed that the r-TCF-1 proteins bound to the CS-T/L probe. However, the same amount of r-TCF-1 did not show any visible binding to the IL4-Lef probe (Fig. 3*B*). Binding of r-TCF-1 to the IL4-Lef probe could be only detected when a higher amount (at least five times more) of r-TCF-1 proteins was added (Fig. 3*C*). Point mutations in the IL4-Lef probe abolished the complex formation, suggesting that binding of TCF-1 to the IL4-Lef probe was sequence-specific (Fig. 3*C*). The complex formed by the IL4-Lef probe was supershifted by the α-TCF-1 but not by the α-LEF-1 antibody, demonstrating that the IL-4 promoter could principally interact with TCF-1, albeit with much lower affinity compared with LEF-1 (Fig. 3*D*). Therefore, in the presence of LEF-1, the IL-4-Lef promoter sequence preferentially binds to LEF-1, as seen in Fig. 3*A*.

β-Catenin itself does not bind to DNA but rather activates target genes by interacting with TCF-1 and LEF-1 (5). Because Jurkat T cells express quite high levels of all three Wnt pathway proteins (Fig. 1*B*), we asked whether β-catenin could co-bind with LEF-1 to the

IL4-Lef probe. To investigate this question, bacterially expressed r-β-catenin protein was added into Jurkat nuclear extracts to increase the β-catenin levels. As a positive control, the CS-T/L probe was used in parallel. The experiment showed that the addition of r-β-catenin proteins into the Jurkat nuclear extracts increased the DNA-protein complex formed by the control CS-T/L probe, and this complex was completely supershifted by the α-β-catenin antibody (Fig. 3*E*, *left panel*). In contrast, the IL4-Lef probe did not show a significant increase in complex formation after the addition of r-β-catenin. Nevertheless, the complex formed by the IL4-Lef probe could be partially supershifted by the α-β-catenin antibody, indicating that β-catenin could interact with LEF-1 on the IL4-Lef probe but with a much lower affinity (Fig. 3*E*, *right panel*).

Because LEF-1 was exclusively found in Th1 cells, we further investigated the tissue specificity of the DNA-protein interactions on the IL-4 LEF-1-binding site using nuclear extracts from the Th1 C29 and Th2 D10 cells. As expected, the nuclear extracts from the Th1 C29 but not from the Th2 D10 cells formed a DNA-protein complex with the IL4-Lef probe (Fig. 3*F*). Equal loadings of the Th1 and Th2 nuclear extracts were controlled by a DNA probe containing the ubiquitously expressed nuclear protein NF-Y. The complex formed by the IL4-Lef probe was supershifted by the α-LEF-1 but not by the α-TCF-1 and α-β-catenin antibodies, demonstrating that this complex was specifically formed by LEF-1 (Fig. 3*G*). Taken together, the above experiments demonstrate that the *IL4* promoter LEF-1/TCF-1 homologous sequence has a strong preference for interaction with LEF-1.

Silencing of LEF-1 Elevates IL-4 Expression

It was recently shown that expression of the Th2 cytokines IL-4, -5, and -13 were strongly suppressed by overexpression of LEF-1 in developing Th2 cells (27). To confirm the negative effect of LEF-1 on the IL-4 expression, we employed a knockdown approach using siRNA in the LEF-1-expressing Jurkat T cells. An approximate 40% down-modulation of the LEF-1 mRNA expression in Jurkat T cells was achieved by using the LEF-1 siRNA (Fig. 4*A*). Corresponding to the reduced mRNA levels, the LEF-1 protein levels were reduced by ~40% (Fig. 4*B*). Subsequently, the cells were analyzed for IL-4 mRNA expression following T cell activation. The siRNA-mediated knockdown of LEF-1 resulted in an ~4-fold increase in the basal level of the IL-4 mRNA expression (Fig. 4*C*). Upon T cell stimulation, 1.5- and 5-fold increases in the inducible IL-4 mRNA expression were seen at 3 and 6 h, respectively (Fig. 4*C*). Thus, in agreement with the LEF-1 overexpression study (27), LEF-1 contributes to negative regulation of the *IL-4* gene.

LEF-1 and TCF-1 Expression Is Down-regulated by IL-4

Differentiation of naïve Th cells into effector cells (Th1 or Th2) during an immune response depends primarily on the cytokine milieu in the periphery (1, 33). IL-4 is the key cytokine that promotes Th2 differentiation. Therefore, we asked whether cytokines, such as IL-4, affect LEF-1 and TCF-1 expression. To investigate this question, purified peripheral blood T cells were treated with IL-4 alone or in combination with T cell activation with PMA and ionomycin or αCD3 plus αCD28. Interestingly, we found that treatment of peripheral blood T cells with IL-4 alone led to an approximate 50% reduction in the TCF-1 mRNA expressions (Fig. 5, *A* and *B*). A subtle reduction in LEF-1 mRNA was also observed in IL-4-treated T cells. As a positive control, the mRNA expression levels of the IL-4-inducible gene SOCS-1 were shown to be increased upon IL-4 treatment (Fig. 5, *A* and *B*). The negative effect of IL-4 on the LEF-1 and TCF-1 mRNA expression was more prominent when the T cells were stimulated with αCD3/αCD28 in the presence of IL-4 (Fig. 5*B*). Correlating with reduced TCF-1 mRNA levels, the protein expression levels of TCF-1 were reduced by ~50% (Fig. 5*C*). Although treatment with IL-4 alone did not show a significant effect on the LEF-1 protein expression level, a combination of IL-4 with T cell stimulation

resulted in complete down-regulation of the LEF-1 protein expression after 8 h of treatment (Fig. 5*C*). To control IL-4 signaling, we showed that STAT6 was phosphorylated upon IL-4 stimulation (Fig. 5*C*). These data indicate that both LEF-1 and TCF-1 expression can be negatively regulated by IL-4 signaling.

DISCUSSION

So far, little is known about the expression and function of Wnt pathway proteins LEF-1 and TCF-1 in mature peripheral T cells. Because LEF-1 and TCF-1 have been found to be expressed in naïve T cells, and their expressions are down-regulated after TCR stimulation (26), it has been speculated that these proteins may have a function in peripheral T cells. Recently, LEF-1 was shown to suppress Th2 cytokine gene expression after introduction into *in vitro* developing Th2 cells (27). Because overexpression of LEF-1 did not prove to significantly affect histone modification at the Th2 cytokine gene loci by chromatin immunoprecipitation assays, the mechanism by which LEF-1 suppresses IL-5 gene expression was suggested by the assumption that LEF-1 interacts with GATA-3 and thereby inhibits DNA binding of GATA-3 to the IL-5 promoter (27). In this study, we confirmed that LEF-1 negatively regulates IL-4 gene expression using a knockdown approach. We have identified a high affinity DNA-binding site for LEF-1 in the IL-4 promoter. We propose that LEF-1 may down-regulate IL-4 gene expression by binding to the negative regulatory element of the IL-4 promoter. Therefore, suppression of the IL-4 gene expression by LEF-1 may occur at two levels: by preventing GATA-3 DNA binding and by negative control of transcription at the negative element of the promoter.

In this study, we show that LEF-1 is preferentially expressed in the non-IL-4-expressing Th1 but not in the IL-4-expressing Th2 cells, indicating that these proteins might also participate in regulation of T cell differentiation. This assumption is supported by the observation that 87% of LEF-1- and/or TCF-1-expressing peripheral T cell lymphomas displayed a Th1-like phenotype. Strikingly, none of the Th2-like peripheral T cell lymphomas expressed LEF-1 and TCF-1 (34). Therefore, it will be interesting to investigate whether LEF-1 is involved in regulation of Th1 polarization. LEF-$1^{-/-}$ mice were reported to have no obvious defects in lymphoid cell populations; however, they die postnatally with multiple developmental abnormalities (20). Thus, a conditional knock-out of LEF-1 in mature T cells is needed to address this question.

We show that LEF-1 binds to the IL-4 promoter with a much higher affinity than TCF-1. This was also found for the LEF-1/TCF-1-binding site on the TCRα enhancer (35). In that study, TCF-1 was shown to be ~10-fold less efficient than LEF-1 in activation of a reporter gene construct under control of the TCRα LEF-1/TCF-1 motif (35). This indicates that LEF-1 might play a more dominant role than TCF-1. However, Van de Wetering *et al.* (35) argued that the abundance of TCF-1 expression in the cell compared with the one of LEF-1 might compensate for its poorer ability in activation of the TCRα enhancer. We also saw that TCF-1 proteins were expressed at much higher levels than LEF-1, particularly in Jurkat T cells (Fig. 1). Nevertheless, TCF-1 did not show detectable binding to the IL-4 promoter probe unless additional recombinant TCF-1 protein was added. Also, TCF-1 proteins do not seem to be expressed in highly differentiated Th1 and Th2 cells. Therefore, we assume that LEF-1 but not TCF-1 plays a major role in regulation of the *IL-4* gene.

In general, LEF-1 and TCF-1 provide sequence-specific binding activity and, in the absence of nuclear β-catenin, collaborate with the transcriptional repressor Groucho and with histone deacetylases to block transcription (5). Wnt signaling leads to an increase of β-catenin in the nucleus, and once in the nucleus, β-catenin associates with LEF-1 and TCF-1 to activate transcription. It has been reported that TCR stimulation may increase nuclear levels of β-

catenin (23). Thus, it might be possible that LEF-1 collaborates with β-catenin to activate the IL-4 promoter. However, our data do not support this possibility. We did not find a co-binding of β-catenin and LEF-1 to the IL-4 promoter probe, although Jurkat T cells express high levels of β-catenin (24) (Fig. 1*A*). A weak co-binding was detected only when additional recombinant β-catenin protein was added into the Jurkat nuclear extracts (Fig. 3*E*). In addition, β-catenin was reported to be expressed at very low or undetectable levels in mature peripheral blood T cells compared with malignant T cells (*e.g.* Jurkat, T cells) (24). We also observed that the Th1 C29 and Th2 D10 cells express very little β-catenin compared with Jurkat T cells (Fig. 1*B*). Therefore, it is unlikely that LEF-1 collaborates with β-catenin to activate the *IL-4* gene.

The cytokine milieu plays a decisive role for naïve $CD4^+$ T cells to differentiate into either a Th1 or Th2 phenotype (1, 33). IL-4 is the key cytokine that promotes Th2 development, whereas IL-12 drives Th1 differentiation. Interestingly, we found that IL-4 negatively regulates the expression of LEF-1 and TCF-1 and thus may further amplify IL-4 expression *via* a positive feedback loop. We did not see, however, a similar effect with IL-12 (data not shown). LEF-1 and TCF-1 were also shown to be down-regulated by IL-15 in $CD8^+$ T cells (26). In that study, IL-15 was demonstrated to shift the balance between stimulatory and inhibitory TCF-1 isoforms in favor of the stimulatory population by preferentially down-regulating the TCF-1 inhibitory isoforms in $CD8^+$ T cells. In contrast, we did not find a clear shift of the balance between stimulatory and inhibitory TCF-1 isoforms in IL-4-treated $CD4^+$ T cells. All of the TCF-1 isoforms were proportionally down-regulated by IL-4 (Fig. 5*C*).

In conclusion, our study provides further evidence that LEF-1 may function as a repressor to control gene expression in peripheral T cells. Particularly, LEF-1 is dominantly expressed in Th1 but not in Th2 cells, implying that this transcription factor might also participate in the regulation of T cell differentiation.

Acknowledgments

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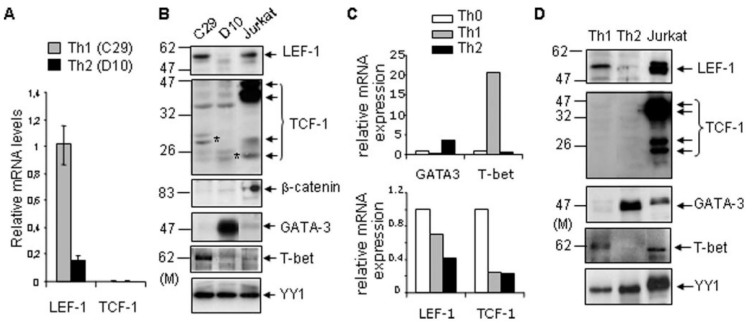

FIGURE 1. LEF-1 is expressed in Th1 but not in Th2 cells

A, LEF-1 mRNA is expressed at a high level in the Th1 clone C29. Total mRNA isolated from C29 and D10 cells was subjected to quantitative PCR analysis. The results are representative of two independent experiments measured in triplicate. *B*, LEF-1 protein is expressed exclusively in the Th1 clone C29. Nuclear proteins isolated from Th1 C29 and Th2 D10 cells were subjected to Western blot analysis. The nuclear extracts from Jurkat T cells, which are known to express all Wnt proteins, were used as controls. YY1 was used as a control for equal loading of proteins. The Th1-specific factor T-bet and the Th2-specific factor GATA-3 were used for control of cell type specificity. The results are representative of four independent experiments. *C*, LEF-1 mRNA is expressed at a higher level in freshly differentiated Th1 than in Th2 cells. Naïve $CD4^+$ T cells were cultured under either Th1 or Th2 differentiation conditions as described under "Experimental Procedures." The mRNA from differentiated cells was subjected to real time PCR analysis for GATA-3, T-bet, LEF-1, and TCF-1. *D*, LEF-1 proteins are expressed dominantly in freshly differentiated Th1 cells. The freshly differentiated Th1 or Th2 cells were subjected to Western blot analysis for LEF-1 and TCF-1. After stripping, the blot was detected for T-bet, GATA-3, and YY1 to control for proper polarization and loading, respectively. The results are representative of two independent experiments.

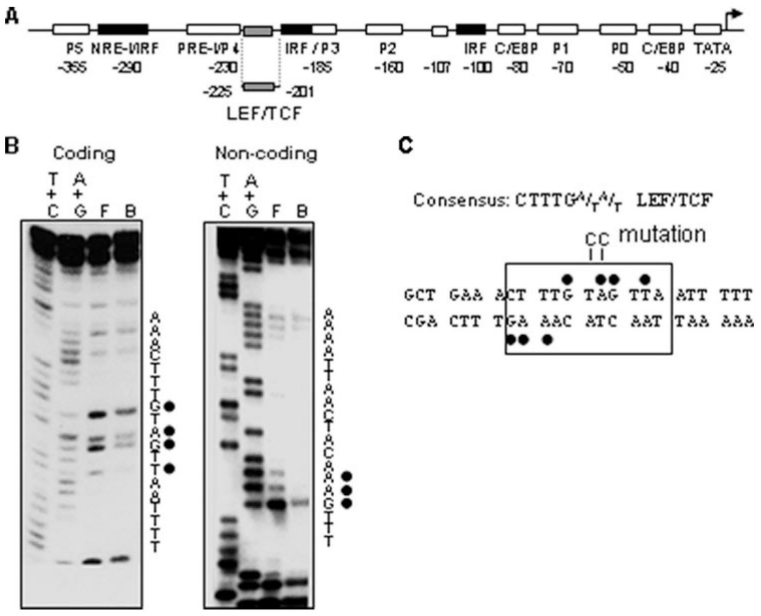

FIGURE 2. IL-4 promoter bears a DNA sequence homologous to the LEF/TCF-binding motif

A, schematic representation of the human IL-4 promoter. The DNA sequence (from −225 to −201) sharing homology to the LEF-1/TCF-1-binding motif is indicated. *B*, specific binding of nuclear proteins to the −225/−201 IL-4 promoter sequence. Nuclear proteins were isolated from Jurkat T cells and subjected to a methylation interference analysis. The free (*lanes F*) and bound (*lanes B*) DNA were analyzed on a sequencing gel. The specific DNA contact sites of nuclear proteins are indicated with *black circles*. *C*, schematic representation of the IL-4 potential LEF-1/TCF-1-binding sequence. The *black circles* indicate the nuclear protein-binding sites detected by methylation interference analysis.

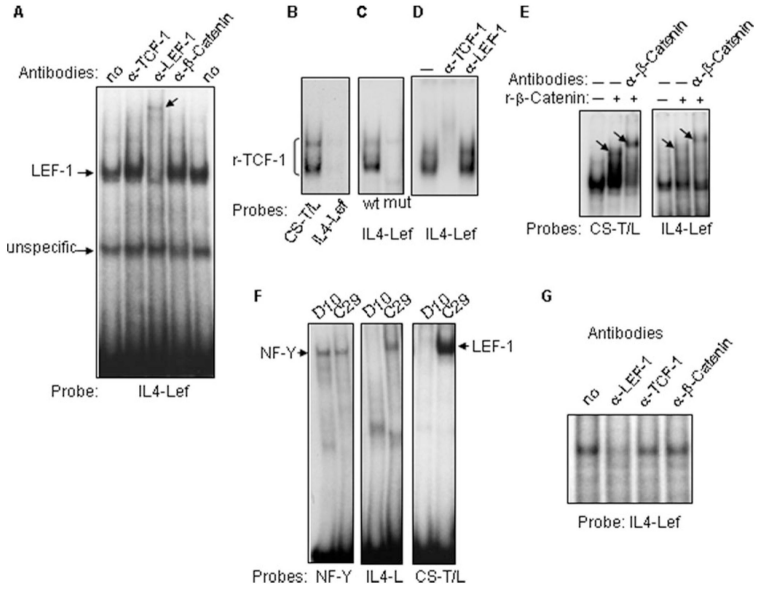

FIGURE 3. The IL-4 −225/−201 promoter sequence is preferentially bound by LEF-1

A, the IL-4 −225/−201 promoter sequence preferentially interacts with LEF-1. The IL-4 −225/−201 DNA sequence was used as a probe (IL4-Lef) in EMSA in the presence or absence of antibodies against LEF-1, TCF-1, and β-catenin. The α-LEF-1 supershifted complex is indicated by an *arrow*. *B*, r-TCF-1 protein preferentially binds to the consensus TCF/LEF motif (CS-T/L). Bacterially expressed r-TCF-1 was used in EMSA with either the IL4-Lef or the CS-T/L (containing the consensus TCF/LEF motif) probe as indicated. *C*, higher amounts of r-TCF-1 were required for detecting its interaction with the IL4-Lef probe. EMSA analysis was carried out with five times more r-TCF-1 proteins than in *B* with the wild-type (*wt*) and the mutated (*mut*, CTTTGCCGTTA instead of CTTTGTAGTTA) IL4-Lef probe. *D*, supershift analysis of the complex formed by the IL4-Lef probe. The IL4-Lef probe was incubated with the r-TCF-1 proteins in the presence or absence of antibodies indicated. *E*, r-β-catenin interacts with both CS-T/L and IL4-Lef probes. EMSA analysis was performed with unclear extracts from Jurkat T cells supplemented with bacterial expressed r-β-catenin proteins. The β-catenin containing complexes detected by the α-β-catenin antibody are indicated by *arrows*. *F*, nuclear extracts prepared from the Th1 C29 but not from the Th2 D10 cells bind to LEF-1 probes. The IL4-Lef and CS-T/L probes were used in EMSA analysis with nuclear extracts from C29 or D10 cells. The NF-Y probe was used to demonstrate equal nuclear extract quality. *G*, antibody analysis of the complex formed by the nuclear proteins from the Th1 C29 cells with the IL4-Lef probe.

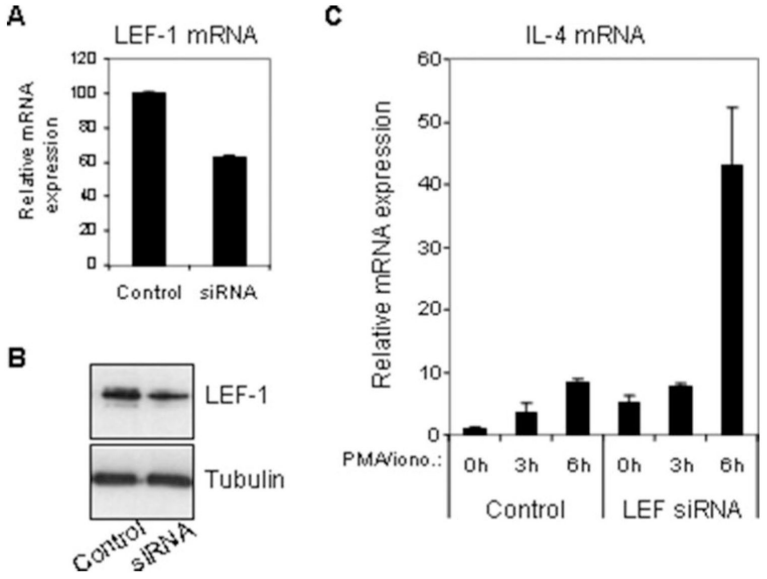

FIGURE 4. Knockdown of LEF-1 increases IL-4 expression

A, down-regulation of LEF-1 mRNA expression by siRNA against LEF-1. Jurkat T cells were transfected with a vector encoding siRNA against LEF-1 or a negative control vector. Positively transfected cells were selected by neomycin resistance, and the effect of LEF-1 siRNA on LEF-1 mRNA expression was monitored by quantitative PCR. The results are representative of four independent transfection experiments measured in triplicate. *B*, down-regulation of LEF-1 protein expression by LEF-1 siRNA. The cells in *A* were subjected to Western blot analysis. *C*, knockdown of LEF-1 resulted in an increase in IL-4 expression. Jurkat T cells positively transfected with either the control or the LEF-1 siRNA were stimulated with PMA (5 ng/ml) and ionomycin (1 μ_M) for the indicated times. The IL-4 mRNA expression levels were analyzed by quantitative PCR. The results are representative of two independent experiments measured in triplicate.

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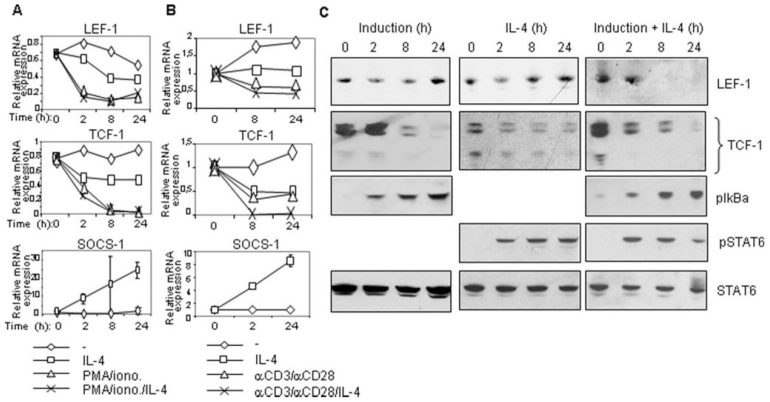

FIGURE 5. IL-4 suppresses expressions of LEF-1 and TCF-1

A and *B*, IL-4 treatment down-regulates mRNA expression of LEF-1 and TCF-1. Freshly isolated primary peripheral blood T cells were treated with IL-4 (50 ng/ml) alone or in combination with PMA/ionomycin (*A*) or αCD3/αCD28 (*B*) stimulation for the indicated times. The LEF-1 and TCF-1 mRNA expression levels were analyzed by quantitative PCR. SOCS-1 mRNA expression levels were used as a positive control for the efficiency of IL-4 stimulation. The results were obtained from three independent experiments with three different donors in *A* and two independent experiments with two different donors in *B*. *C*, IL-4 treatment down-regulates protein expression levels of LEF-1 and TCF-1. The treated T cells in *A* were further analyzed by Western blot with specific antibodies against LEF-1 and TCF-1. Levels of phosphorylated STAT6 (pSTAT) and total STAT6 were used as controls for the efficiency of IL-4 stimulation and equal loadings. Levels of phosphorylated IκBα were examined for controlling the efficiency of T cell activation.

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