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KLF13 Cooperates with c-Maf To Regulate IL-4 Expression in CD4+ T Cells

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Abstract

Kruppel-like factor (KLF) 13 is a transcription factor that positively regulates expression of the chemokine RANTES 3–5 d after activation of T cells. In this study, we document a key role for KLF13 in the expression of IL-4 in $CD4^+$ T cells. Gene expression analysis in activated T cells from *Klf13*$^{-/-}$ mice showed that IL-4, along with other Th2 cytokine genes, was downregulated when compared with cells from wild-type mice. The decreased levels of IL-4 were not associated with changes in expression of the Th2-inducing transcription factors GATA3 or c-Maf. Additional analysis revealed that KLF13 directly binds to IL-4 promoter regions and synergizes with c-Maf to positively regulate IL-4 expression. These results indicate that KLF13 is a positive regulator for differentiation of Th2 cells, as part of the transcriptional machinery that regulates IL-4 production in Th2 cells.

$CD4^+$ T cells mediate a variety of critical roles in adaptive immune responses. Once triggered by Ag and cytokines, naive $CD4^+$ T cells can differentiate into at least four different subsets: Th1, Th2, Th17, and induced T regulatory cells (1). Th1 cells are critical for immune responses against intracellular pathogens and produce IFN-γ, TNF-α, and IL-2. Th2 cells are involved in humoral immunity as well as the development of allergy and other allergic diseases through the production of a variety of cytokines, including IL-4, IL-5, IL-9, IL-10, IL-13, IL-21, and IL-25 (1). Th17 cells can produce IL-17, which has a critical role in autoimmune diseases and inflammation (2), whereas regulatory T cells suppress the immune response via IL-10 and TGF-β1 (3).

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Disclosures

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IL-4 is a pleiotropic cytokine produced by $CD4^+$ T cells, NKT cells, basophils, and mast cells (4). IL-4 functions as an initiator of Th2 cell development, a regulator for MHC class II expression, an inducer of Ab isotype switching in B cells, and a modulator of B cell growth (5–7). IL-4 autoregulates its own expression through its receptor and several transcription factors (1, 8). IL-4 binds to the IL-4R α-chain (IL-4Rα), resulting in the phosphorylation and activation of STAT6. Phosphorylated STAT6 then translocates into the nucleus and—together with TCR signaling and transcription factors, including GATA3, c-Maf (9–11), and NFAT (8, 12)—regulates IL-4 expression (13–15). This autocrine effect can also regulate Th2 cell differentiation via production of other cytokines such as IL-5 and IL-13 (8).

GATA3 and c-Maf are Th2-specific transcription factors. GATA3 has been called a master regulator for development of Th2 cells because it binds to loci of several genes required for Th2 development and function (16–18). c-Maf is a member of the basic-region leucine-zipper (b-zip) protein and was the first transcription factor identified as a regulator of Th2 cell differentiation (9). Together with IFN regulatory factor (IRF) 4 and NFAT, c-Maf promotes IL-4 expression (19) and positively regulates IL-2R α-chain expression (11, 20) in Th2 cells. Recently, it was reported that c-Maf contains three tyrosines whose phosphorylation is important for IL-4 production (21).

Kruppel-like factors (KLFs) are a subclass of zinc finger transcription factors that play important roles in different aspects of cell growth, development, and differentiation. KLFs have a DNA binding domain containing three C_2H_2-type zinc fingers that bind to either a CACCC element or a GC box in the proximal promoter of targeted genes. Our group have identified KLF13 as an essential transcription factor for the late expression of chemokine RANTES in T lymphocytes (22). KLF13 also acts as a negative regulator for the antiapoptotic protein BCL_{XL} (23) and positively regulates the expression of *Rcn3* (BC025602) and *Eomesodermin* (Eomes) in naive $CD8^+$ T cells (24). However, other transcriptional targets of KLF13, as well as its role in T cell lineage determination, have not been fully elucidated.

To address this issue, we sought to identify genes that are regulated by KLF13 in mouse $CD4^+$ T cells. Using microarray analysis, we compared gene expression in activated $CD3^+$ T lymphocytes from wild-type (WT) and *Klf13*$^{-/-}$ animals. Importantly, several Th2 cytokines, including IL-4, were among the genes found to be misregulated in *Klf13*$^{-/-}$ $CD3^+$ lymphocytes. Our studies revealed that KLF13 enhances IL-4 production via at least two mechanisms: it binds directly to the IL-4 promoter, and it interacts with the Th2-specific transcription factor c-Maf. Thus, KLF13 is a new member of the transcription factor family that regulates the expression of IL-4 in Th2 cells.

Materials and Methods

Mice and cell culture conditions

Klf13$^{-/-}$ mice on a BALB/c background were generated and maintained as previously described (24). All animal studies were reviewed and approved by the Animal Care Committee of the National Cancer Institute. EL4 cells were cultured in RPMI 1640 supplemented with 10% FBS and 100 U/ml penicillin/streptomycin at 37°C in 95%

humidity and 5% CO_2. Primary murine T cells were cultured in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin/streptomycin, and 55 μM 2-mercaptoethanol at 37°C in 95% humidity and 5% CO_2.

cDNA microarrays and quantitative RT-PCR

$CD3^+$ T cells were isolated using the EasySep Negative Selection Mouse T Cell Enrichment Kit (STEMCELL Technologies, Vancouver, Canada) from spleens of five WT or *Klf13*$^{-/-}$ mice. Isolated cells (3–5 × 10^6/ml) were cultured for 4 d on tissue culture plates precoated with 3 μg/ml anti-mouse CD3 Ab (BioLegend, San Diego, CA) and 3 μg/ml soluble anti-mouse CD28 Ab (BioLegend). On day 4, cells were harvested and total RNA was isolated using the RNeasy Kit (QIAGEN, Valencia, CA). Microarray analysis was performed at the Laboratory of Molecular Technology at the Frederick National Laboratory for Cancer Research (Frederick, MD), using Affymetrix GeneChip Mouse Gene St 1.0 ST. Microarray data were analyzed using Partek Genomics Suite software (Partek, St. Louis, MO, $p < 0.05$ and fold > 1.5). Microarray data are available using the GEO accession number GSE50617 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM1224728).

For quantitative RT-PCR, cDNA was synthesized from 1 μg total RNA using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA), and the cDNA was then used for amplification with Power SYBER Green PCR Master Mix (Applied Biosystems/Life Technologies, Grand Island, NY) in a 7900HT Fast Real-Time PCR System (Applied Biosystems). The primers used for validation were as follows: murine (m)IL-4 (forward: 5′-TGAACGAGGTCACAGGAGAA-3′, reverse: 5′-CGAGCTCACTCTCTGTGGTG-3′); mIL-5 (forward: 5′-GCAATGAGACGATGAGGCTT-3′, reverse: 5′-CCCACGGACAGTTTGATTCT-3′); mIL-9 (forward: 5′-TGACCAGCTGCTTGTGTCTC-3′, reverse: 5′-TATCCTTTTCACCCGATGGA-3′); mIL-10 (forward: 5′-ATCGATTTCTCCCCTGTGAA-3′, reverse: 5′-TGTCAAATTCATTCATGGCCT-3′); mIL-13 (forward: 5′-TGTGTCTCTCCCTCTGACCC-3′, reverse: 5′-CGAGCTCACTCTCTGTGGTG-3′); mc-Maf (forward: 5′-GATGGCTTCAGAACTGGCA-3′, reverse: 5′-CATGAAAAATTCGGGAGAGG-3′); mGATA3 (forward: 5′-GCCTGCGGACTCTACCATAA-3′, reverse: 5′-AGGATGTCCCTGCTCTCCTT-3′); mβ-actin (forward: 5′-GTCCACCCGCGAGCACAGCTT-3′, reverse: 5′-CTTTGCACATGCCGGAGCCGT-3′).

In vitro Th2 cell differentiation

$CD4^+$ T cells were isolated with EasySep Mouse $CD4^+$ T Cell Enrichment Kit (STEMCELL Technologies) and cultured on plates precoated with 3 μg/ml anti-mouse CD3 Ab and supplemented with 3 μg/ml soluble anti-mouse CD28 Ab. For Th2 polarization, cultures were also supplemented with 10 μg/ml anti–IL-12/23 Ab, 10 μg/ml anti–IFN-γ Ab, and 25 ng/ml rIL-4. All reagents were purchased from BioLegend. For analysis of IL-4Rα expression, purified splenic $CD4^+$ T cells were cultured with immobilized anti-CD3 and soluble anti-CD28 for 2 d, washed, and cultured overnight in fresh medium. Cells were then cultured in the presence or absence of rIL-2 for 4 h (25) and analyzed by FACS.

Plasmids and site-directed mutagenesis

The constructs pcDNA 3.1 V5-tagged mouse Klf-13 (26) and pCMV FLAG-tagged c-Maf were used in this study. Two regions of the IL-4 upstream promoter (−1 ~ −500 bp and −1 ~ −234 bp) were amplified with PCR using AccuPrime Pfx DNA polymerase (Invitrogen, Carlsbad, CA) and subcloned into the pGL4.12 vector (Promega, Madison, WI) for generating pGL4.12 IL-4 0.5 kb and pGL4.12 IL-4 0.23 kb. PCR-based site-directed mutagenesis was performed on the KLF13 binding site (pGL4.12 0.5 kb mutant) and c-Maf binding site (MU1, MU2, and MU3) using Phusion High-Fidelity DNA Polymerase and DpnI enzyme (New England Biolabs, Ipswich, MA). The pGL4.12 IL-4 0.5 kb was used as the template for KLF13 mutant, and pGL4.12 IL-4 0.23 kb was used as the DNA template for MU1 and MU2. For MU3, MU2 was used as the template. The primers used in this study were as follows: IL-4 promoter luciferase 0.5 kb (forward: 5′-AAAAAACTCGAGAGAGTTTCCAAGGGGCCC-3′, reverse: 5′-TTTTTTAGATCTCAATAGCTCTGTGCCGTCAG-3′); IL-4 promoter luciferase 0.5 kb mutant (forward: 5′-CCAGGGCGACACCAGCATACTCGGACACCTGTGAC-3′, reverse: 5′-GTCACAGGTGTCCGAGTATGCTGGTGTCGCCCTGG-3′); IL-4 promoter luciferase 0.23 kb (forward: 5′-AAAAAACTCGAGTGGTCTGATTTCACAGGAAAA-3′, reverse: 5′-TTTTTTAGATCTCAATAGCTCTGTGCCGTCAG-3′); IL-4 promoter luciferase 0.23 kb MU1 (forward: 5′-CATTTTCCCTTGGTTGACTCAACTTTAACTCTATAT-3′, reverse: 5′-ATATAGAGTTAAAGTTGAGTCAACCAAGGGAAAATG-3′); IL-4 promoter luciferase 0.23 kb MU2 (forward: 5′-CTCCTGGAAGAGAGGATTAGATTGGCCCAGAATAAC-3′, reverse: 5′-GTTATTCTGGGCCAATCTAATCCTCTCTTCCAGGAG-3′); and IL-4 promoter luciferase 0.23 kb MU3 (forward: 5′-CATTTTCCCTTGGTTGACTCAACTTTAACTCTATAT-3′, reverse: 5′-ATATAGAGTTAAAGTTGAGTCAACCAAGGGAAAATG-3′).

Transfection and luciferase assay

For transfection, 4×10^6 EL4 cells were transiently transfected with 5 μg plasmid DNA using the Amaxa Cell Line Nucleofactor Kit V (Lonza, Walkersville, MD) and pRL-SV40 *Renilla* luciferase was used as a control vector (Promega). After transfection, cells were cultured with 10 ng/ml PMA and 1 μM ionomycin for 14 h, and luciferase activity was analyzed with the Dual-Luciferase Reporter Assay System (Promega). Data were normalized using Renilla levels from cotransfected pRL-SV40 control vector.

Intracellular staining and flow cytometry

All Abs for FACS were purchased from BD Biosciences (San Jose, CA) and BioLegend. To detect cytokine production by flow cytometry, cells were restimulated with PMA/ionomycin for 4 h in the presence of monensin or brefeldin, fixed and permeabilized using the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences), and stained following the manufacturer's instructions. Fluorescence signals were collected using a FACSCalibur flow cytometer or an LSRFortessa cell analyzer (BD Biosciences) and analyzed using FlowJo software (TreeStar, Ashland, OR).

Western blot analysis

Abs for KLF13, c-Maf, and α-actinin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and GAPDH was purchased from Millipore (Billerica, MA). Mouse anti-V5 Ab was purchased from Invitrogen (Carlsbad, CA). Rabbit anti-FLAG Ab was purchased from Cell Signaling (Danvers, MA). For Western blot analysis, cells were lysed in cell lysis buffer (150 mM NaCl; 1 mM EDTA; 1 mM ethylene glycol-bis(β-aminoethyl ester)-*N,N,N′,N′*-tetraacetic acid; 1% Triton; 2.5 mM sodium pyrophosphate; 1 mM β-glycerolphosphate; 1 mM Na_2VO_4, in 20 mM Tris, pH 7.4) with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Rockford, IL) and incubated on ice for 30 min. After centrifugation, the supernatant proteins (containing 25–50 μg protein) were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Appropriate primary Abs and secondary Abs were used for Western blots.

Coimmunoprecipitation

EL4 cells (5×10^6 cells) were transiently transfected with 5 μg pCDNA3.1-Klf13-V5 and 5 μg pCMV-FLAG-c-Maf, using the Neon Transfection System (Invitrogen). Specific settings for EL4 cells were 1080 V for a duration of 50 ms and 1 pulse. Transfected cells were cultured overnight and stimulated with PMA/ionomycin for 1 h. After stimulation, cells were harvested and lysed in RIPA buffer (150 mM NaCl; 10 mM Tris, pH 7.2; 0.1% SDS; 1.0% Triton X-100; 1% deoxycholate; 5 mM EDTA; and protease and phosphatase inhibitor mixture). The cell lysates were immunoprecipitated using anti-V5 (Klf13) or anti-FLAG (c-Maf) Abs, respectively, subjected to SDS-PAGE, and immunoblotted with anti-FLAG (c-Maf) or anti-V5 (Klf13). Normal IgG was used as the negative control.

Chromatin immunoprecipitation

Briefly, 1×10^7 Th2 polarized mouse $CD4^+$ T cells were cross-linked with 1% formaldehyde and lysed (PBS, 0.5% Triton X-100, and protease and phosphatase inhibitor mixture). Chromatin was sheared using a Sonicator Q500 (Qsonica, Newtown, CT) and immunoprecipitated with anti-KLF13 Ab or control normal IgG. The recovered DNA samples were analyzed using RT-PCR. The primers for chromatin immunoprecipitation (ChIP) were as follows: IL-4 ChIP1 (forward: 5′-ACTTTCTTGATATTACTCTGTCTTTCC-3′, reverse: 5′-GTTGCCACTGGCTCTCCTC-3′) and IL-4 non (forward: 5′-CATGGTCAGCTTCCTCTGCT-3′, reverse: 5′-CTTTGGTCCCCACACTTGTT-3′).

Small interfering RNA transfections

Mouse $CD4^+$ T cells were cultured on plates precoated with 3 μg/ml anti-mouse CD3 Ab and supplemented with 3 μg/ml soluble anti-mouse CD28 Ab. After overnight activation, mouse $CD4^+$ T cells were transfected with either a nontargeting control or Klf13 targeting small interfering (siRNA) (Dharmacon/Thermo Fischer Scientific) using the Neon Transfection System (Invitrogen). Cells were harvested for expression analysis at 48 h posttransfection.

Chemiluminescent EMSA

A nonradioactive chemiluminescent EMSA kit (Active Motif, Carlsbad, CA) was used to examine whether mouse Klf13 binds to the putative binding site on the mouse IL-4 proximal promoter. The oligonucleotides used as probes in gel shift assays were end-labeled at their 5′ ends with biotin, and the sequences are as follows: RANTES: forward, 5′-GCTATTTTGGAAACT CCCCTT-3′; reverse, 5′-AAGGGGAGTTTCCAAAATAGC-3′; IL-4: forward, 5′-GACACCAGCACCCTCGGACAC-3′; reverse, 5′-GTGTCCGAGGGTGCTGGTGTC-3′. Competitor oligonucleotides were the same sense and antisense sequences as above without the 5′-biotin label. The 21-bp probe and competitor DNA consisted of cDNA fragments annealed by heating to 95°C and slowly cooled to room temperature. All EMSA experiments were performed on 5% polyacrylamide gels in 0.5× Tris-borate-EDTA buffer. Each EMSA reaction mixture contained 50 ng poly(deoxyinosinic-deoxycytidylic) acid, 1× LightShift EMSA Kit binding buffer, 20 fmol biotin-labeled DNA probe and 5 μg 293T cell nuclear extract overexpressing mouse Klf13, and 4 pmol nonbiotinylated competitor DNA. EMSA gels were electroblotted onto GeneScreen Plus Hybridization Transfer Membrane (PerkinElmer). Signal development followed the LightShift Chemiluminescence EMSA Kit protocol with HyBlot ES Autoradiography Film (Denville Scientific) for luminescence detection.

Statistics

All experiments were performed at least three times. Data were analyzed using the Student *t* test and $p < 0.05$ was considered statistically significant.

Results

Klf13 affects expression of Th2 genes in CD3$^+$ T cells

To identify genes potentially regulated by KLF13 in T cells, we performed cDNA microarray analysis on activated CD3$^+$ T cells from WT and *Klf13*$^{-/-}$ mice. mRNAs encoding IL-4, IL-5, IL-9, IL-10, and IL-13, cytokines characteristic of Th2 cells, were all significantly decreased in cells from *Klf13*$^{-/-}$ mice, and these results were confirmed by real-time RT-PCR (Table I). These findings implicate *Klf13* in the differentiation of Th2 cells. Because of the critical role of IL-4 in the induction of other Th2-type cytokines and in the polarization of CD4$^+$ Th2 cells (8), we focused on a potential direct transcriptional regulation of IL-4 by Klf13.

Splenocytes and lymph node cells obtained from WT or *Klf13*$^{-/-}$ mice were stimulated with anti-CD3 and anti-CD28 in vitro and stained for intracellular expression of IL-4 after 4 d of culture. As expected, the number of IL-4–expressing cells was reduced in *Klf13*$^{-/-}$ mice, compared with IL-4–expressing cells from WT spleen or lymph node (Fig. 1A). Next, we purified CD4$^+$ T cells and cultured them under Th2 polarizing conditions. The number of cells expressing IL-4 (Fig. 1B) and IL-4 mRNA (Fig. 1C) was significantly decreased in CD4$^+$ cells from *Klf13*$^{-/-}$ mice, compared with cells from WT animals. Decreased IL-4 expression was also observed after knockdown of KLF13 in CD4$^+$ T cells from WT animals (Fig. 1D), indicating that the effect of KLF13 on IL-4 expression is post–T cell development.

Absence of KLF13 does not affect the expression of Th2-inducing transcription factors or the IL-4Rα

To assess the potential mechanism by which KLF13 regulates IL-4 expression, we checked the expression level of some known Th2-specific transcription factors, GATA3 and c-Maf, in Th2 polarized $CD4^+$ T cells. No significant differences in GATA3 and c-Maf mRNA (Fig. 2A, 2B) or protein (Fig. 2C, 2D) were detected in WT and *Klf13*$^{-/-}$ cells. Leonard and coworkers (25) observed that IL-4 expression is also regulated by modulation of IL-4Rα levels. IL-4Rα is expressed at low levels in resting T cells (27) and is upregulated after activation and in response to both IL-2 and IL-4 (25). We therefore checked IL-4Rα levels by flow cytometry, using the method described by Leonard and coworkers. $CD4^+$ cells from WT and *Klf13*$^{-/-}$ mice were activated for 2 d by immobilized anti-CD3 plus soluble anti-CD28. Cells were then washed, resuspended in fresh medium, and cultured overnight. rIL-2 was added for 4 h, and IL-4Rα levels were measured. No differences in IL-4Rα expression were detected, regardless of whether cells were obtained from WT or *Klf13*$^{-/-}$ mice (Fig. 2E). These results suggest that KLF13 regulates IL-4 via direct binding to the IL-4 promoter and/or by cooperating with another transcription factor that binds to the IL-4 promoter.

KLF13 directly binds to the IL-4 promoter and promotes IL-4 expression

KLF family transcription factors have three conserved zinc finger motifs that bind to GC-rich sequences or to a CACCC box in vitro (28). Within the ~500-bp region upstream of the IL-4 transcription start site, we found a CACCC box at −338 bp (Fig. 3). We performed luciferase reporter assays using a construct containing 500 bp upstream of the start site (pGL4.12 IL-4 0.5 kb) to analyze KLF13-dependent promoter activity. IL-4 promoter activity was increased by cotransfection of KLF13 in a concentration-dependent manner (Fig. 4A). To validate binding of KLF13 to the CACCC box at −338 bp, we generated another construct containing a mutation in the CACCC box (CACCC→CATAC) (29). As expected, the mutant construct showed significantly reduced luciferase activity (Fig. 4B). To confirm the binding of KLF13 to the IL-4 promoter, ChIP assays were conducted in Th2 polarized mouse $CD4^+$ T cells, using primers designed to detect the region around the CACCC box. We observed significantly enhanced binding of KLF13 to this region, using anti-KLF13 Ab compared with IgG (Fig. 4C). Binding of KLF13 to the IL-4 promoter was independently confirmed by gel shift (Fig. 4D). Incubation of KLF13 with either a 21-bp biotin-labeled probe from the RANTES promoter or a similarly sized biotin-labeled probe from the IL-4 promoter produced a slower migrating band, whereas inclusion of 200-fold excess of unlabeled probe led to elimination of the shifted bands. Collectively, these results indicate that KLF13 directly binds to the IL-4 promoter and positively regulates IL-4 expression.

KLF13 also regulates IL-4 expression by interacting with c-Maf

To confirm that the KLF13 binding site at −338 bp is indeed a functional KLF13 transcription site, we generated an IL-4 promoter luciferase reporter construct containing 0.23 kb upstream of the IL-4 start site (pGL4.12 IL-4 0.23 kb). This construct lacks the potential KLF13 binding sequence at −338 bp. Of interest, there was a small but reproducible increase in luciferase activity of this construct when KLF13 was cotransfected

along with the reporter construct (Fig. 5A). This result was consistent with using the pGL4.12 IL-4 0.5 kb mutant and suggested either that additional KLF13 binding motifs exist in this truncated promoter region or that KLF13 can synergize with other factors to activate IL-4. To investigate possible cooperation with other factors, we focused on c-Maf because of its selective effect on IL-4 production in Th2 cells. We identified two potential c-Maf binding sites, known as Maf recognition elements (MARE), (Fig. 3) in the region 150 bp upstream of the IL-4 transcription start site. To study a potential functional interaction between KLF13 and c-Maf on the IL-4 promoter, we made IL-4 promoter luciferase reporter mutant constructs lacking either (MU1 and MU2) or both (MU3) of the MARE sites (Fig. 5B). EL4 cells were transfected with these constructs along with KLF13 and/or c-Maf. Transfection of c-Maf in the absence of KLF13 caused a significant increase in luciferase activity in the IL-4 promoter constructs that contained either or both MARE sites, but did not cause significant activity in the construct lacking both MARE sites. Cotransfection of KLF13 along with c-Maf significantly increased luciferase activity in the construct containing both MARE sites as well as the MU1 construct that contains the second MARE site, but did not enhance activity in the MU2 construct that contains the first MARE site (Fig. 5C). This result demonstrates that KLF13 functionally interacts with c-Maf bound to the second MARE site in the IL-4 promoter. To confirm a physical interaction between KLF13 and c-Maf, we performed coimmunoprecipitation studies in EL4 cells. Indeed, KLF13 can physically interact with c-Maf when both proteins were overexpressed in EL4 cells (Fig. 5D). Thus, KLF13 functionally cooperates with C-Maf to enhance expression of IL-4.

Discussion

Differentiation of naive $CD4^+$ T cells into Th2 cells is a complex process initiated by the interaction of the TCR with cognate Ag and by cytokine-mediated signaling. IL-2 activates STAT5, which is critical for T cell proliferation and differentiation, whereas IL-4 activates STAT6, leading to upregulation of the transcription factor GATA3, termed the master regulator of Th2 cells. GATA3, STAT5, and STAT6 are indispensable for Th2 differentiation, and a variety of other transcription factors are also very important for IL-4 production. We show in this article for the first time, to our knowledge, that KLF13, a member of the Kruppel-like family of transcription factors, plays a central role in expression of IL-4 by Th2 cells.

A number of transcription factors act directly on the IL-4 promoter, mainly within the 300 bp upstream of the transcription start site. NFAT1, NF-κB, C/EBPβ, IRF4, CP2, c-Maf, JunB, nuclear transcription factor Y, and Yin-Yang 1 are all positive regulators, whereas IRF1, IRF2, class II transactivator, NFAT4, and HMGI(Y) are negative regulators (30). In addition, IL-4 produced by other cells, such as mast cells, basophils, NKT cells, and memory Th2 cells, enhances IL-4R expression and upregulates GATA-3, providing a powerful positive feedback loop for Th2 differentiation and IL-4 expression.

Members of the KLF family are involved in the activation, differentiation, and migration of T cells (28). KLF13 acts as a positive regulator for RANTES expression (22), a negative regulator of the antiapoptotic protein $BCL\text{-}X_L$ (23), and a regulating factor for the

expression of *Rcn3* (BC025602) and Eomes (24). However, other target genes regulated by KLF13 in activated T cells are still unknown. Our microarray analysis shows that KLF13 is involved in regulation of several genes associated with the immune response, including cytokines, signaling components, and cytotoxic proteins. The finding that activated T cells from mice lacking KLF13 are deficient in expression of IL-4 as well as IL-5, IL-9, IL-10, and IL-13 was somewhat surprising. Inspection of the proximal promoter regions of these genes revealed that IL-4, IL-5, IL-10, and IL-13 all contain putative KLF13 binding sites.

c-Maf is a member of the basic and leucine zipper domain (bZIP) transcription factor family. The leucine zipper is located near the C terminus, is highly homologous to a similar domain in AP-1 and CREB/ATF proteins, and facilitates homo- and heterodimerization (31). In addition, c-Maf has an adjacent region termed the ancillary DNA binding domain that mediates DNA binding (32). The mouse IL-4 promoter contains two potential MARE. The first is a half MARE binding site (TCAGCA, residues −42 to −37) immediately downstream of the promoter. This site was initially described by Glimcher and coworkers as the c-Maf binding region in the IL-4 promoter (33, 34). The second is a different half MARE binding site farther upstream (TGCTGA) at residues −108 to −116. Mutation of either MARE site reduced the reporter gene activity of the IL-4 promoter construct transfected into EL-4 cells with c-Maf by ~50%, and mutation of both sites reduced the activity to basal levels. This finding indicates that both sites are operative in the IL-4 promoter. However, synergy between KLF13 and c-Maf was observed only when the site at −42 to −37 remained intact, suggesting that the c-Maf/KLF13 complex preferentially binds to that site. It has been reported that c-Maf interacts with other transcription factors, including Sox9, Foxp3, and Pax-6 (35–37), and our previous report showed that KLF13 can bind the RANTES promoter and that it interacts with the transcription factors NF-κB p50, NLK, p300/CBP, and PCAF (38). We show here that c-Maf and KLF13 physically interact and that c-Maf/KLF13 regulates IL-4 expression. The IL-4 promoter contains potential binding sites for other transcription factors, including NF-κB, NFAT, and STATs, but we focused on c-Maf because of its specific role in IL-4 production and Th2 differentiation. We cannot exclude the possibility that KLF-13 also interacts with other transcription factors to affect IL4 expression. In addition, we identified MARE sites in the IL-5, IL-10, and IL-13 promoters, but not in the RANTES, Rcn3, or EOMES promoters.

A role for KLF family members in Th2 differentiation has not been well characterized. KLF10 transduced cells showed low expression of the Th2 master regulator GATA3, suggesting a potential negative role in the regulation of Th2 immune response. Our data demonstrate that KLF13 is a positive regulator of IL-4 expression in T cells. KLF family members bind to a consensus DNA binding motif, CACCC. However, it is possible that individual KLFs can interact with DNA binding motifs that deviate from this consensus binding sequence. We observed a small but reproducible reporter gene response, using the IL-4 promoter containing a mutated CACCC motif. One interpretation of this result is that KLF13 can bind to other, yet-to-be-described sequences in addition to the canonical CACCC motifs. Using ChIP followed by DNA sequencing studies, we identified other sequences bound by KLF13 in human lymphocytes (W. Zhang, A.M. Krensky, and C. Clayberger, unpublished observations). The results obtained from this ChIP–DNA sequencing study

could potentially provide key answers about KLF13-specific binding motifs at the whole genome.

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Abbreviations used in this article:

ChIP	chromatin immunoprecipitation
IL-4Rα	IL-4R α-chain
IRF	IFN regulatory factor
KLF	Kruppel-like factor
m	murine
MARE	Maf recognition element
siRNA	small interfering RNA
WT	wild-type

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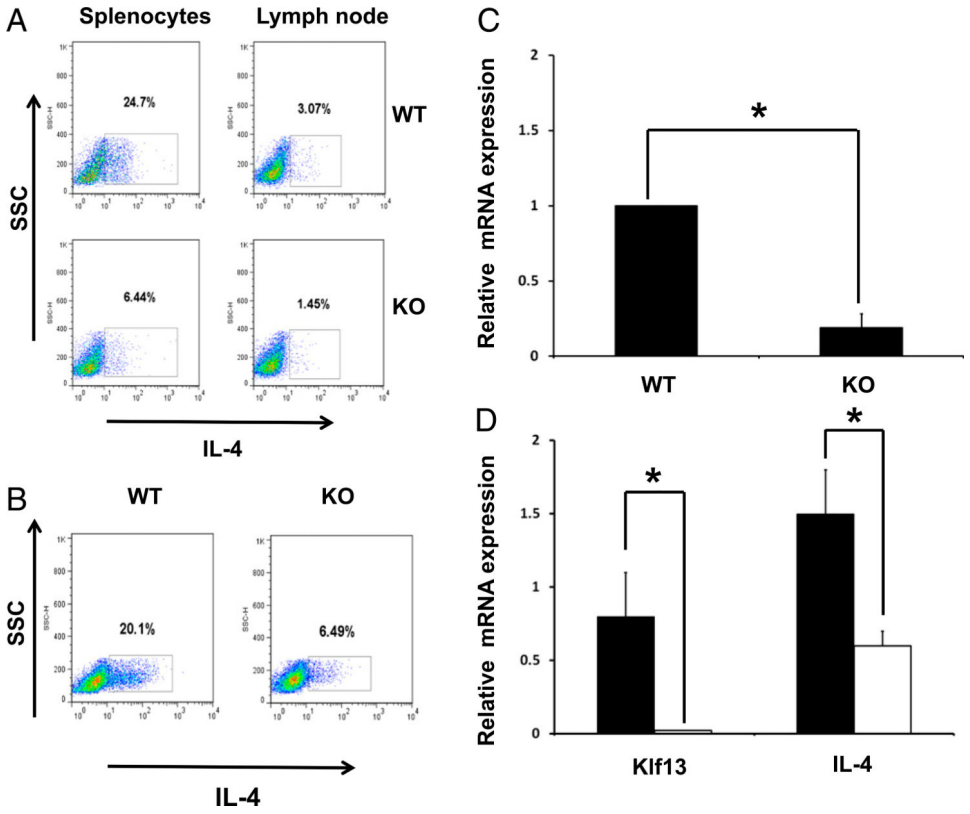

FIGURE 1.

IL-4 expression is strongly reduced in the absence of KLF13. (**A**) Splenocytes and lymph node cells from WT and *Klf13*$^{-/-}$ animals were activated with anti-CD3 plus anti-CD28 for 4 d and restimulated with PMA/ionomycin; IL-4 expression was then determined by FACS. (**B**) CD4^{+} T cells from WT and *Klf13*$^{-/-}$ animals were cultured under Th2 polarizing conditions for 7 d and restimulated with PMA/ionomycin, and IL-4 expression was then determined by FACS. (**C**) Reduced IL-4 expression in *Klf13*$^{-/-}$ T cells is due to reduced levels of IL-4 mRNA. (**D**) siRNA knockdown of KLF13 in activated mouse CD4^{+} T cells from WT mice results in decreased expression of IL-4 mRNA. Black bars, nontargeting control siRNA; open bars, KLF13 siRNA. All experiments are representative of three independent replicates. *$p < 0.05$.

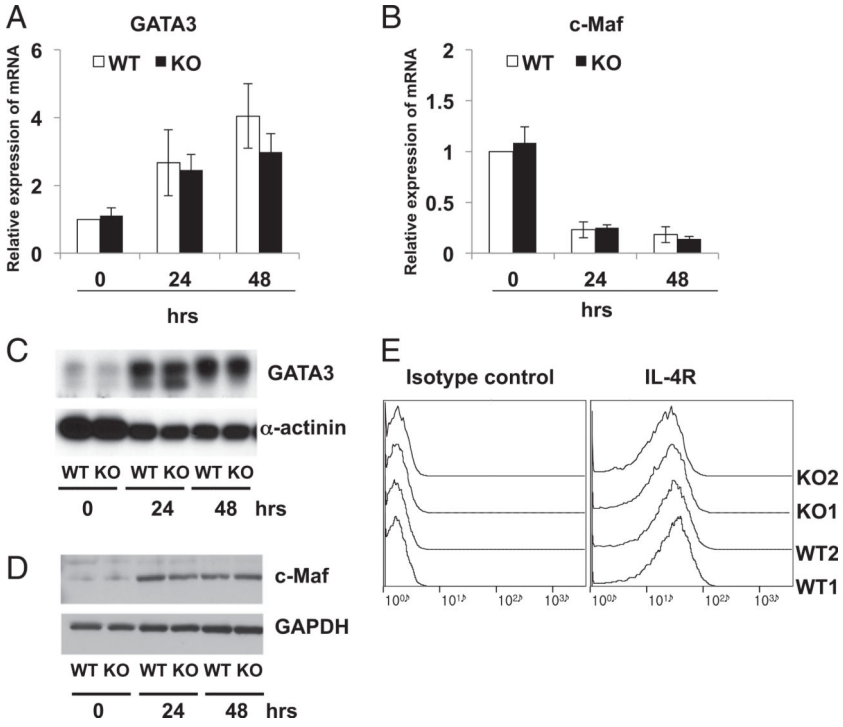

FIGURE 2.

Expression of GATA-3, c-Maf, and IL-4Rα is similar in WT and *Klf13*$^{-/-}$ T cells. (**A** and **B**) CD4^{+} T cells from WT and *Klf13*$^{-/-}$ mice were activated with anti-CD3 and anti-CD28 and analyzed for mRNA levels of GATA3 and c-Maf by quantitative RT-PCR. (**C** and **D**) Protein levels of GATA3 and c-Maf from the same samples were detected by Western blot. (**E**) IL-4Rα expression in activated CD4^{+} T cells from WT and *Klf13*$^{-/-}$ mice. All experiments are representative of at least three independent replicates.

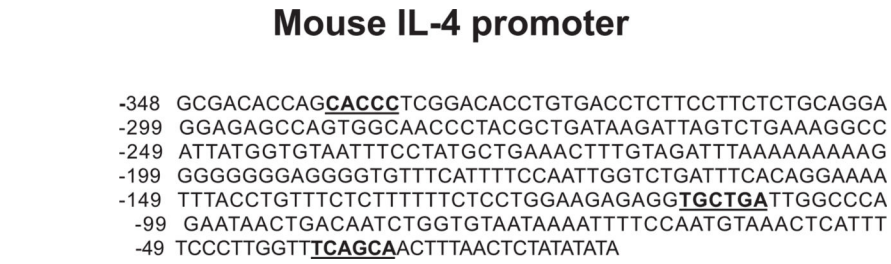

FIGURE 3.

DNA sequence of the immediate upstream region of the IL-4 promoter. The underlined CACCC sequence is the potential KLF13 binding site; underlined TGCTGA (MARE1) and TCAGCA (MARE2) are the potential c-Maf binding element sites.

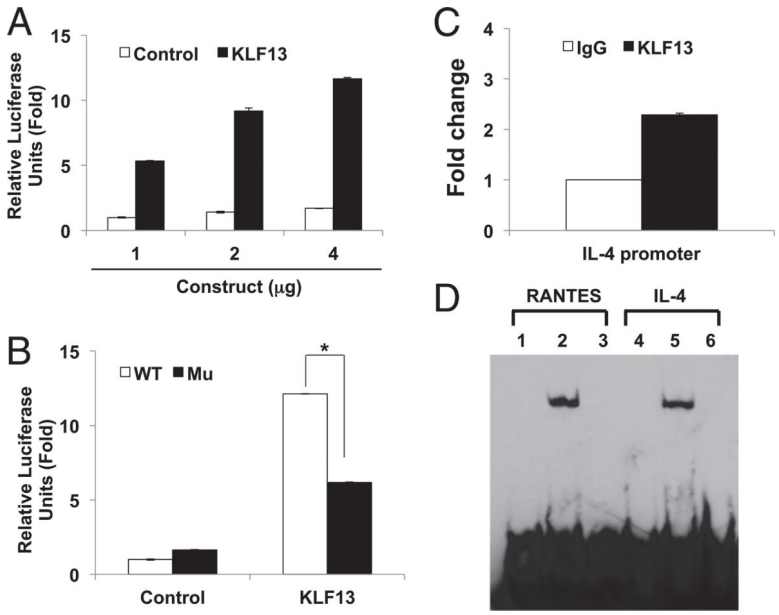

FIGURE 4.

KLF13 binds directly to the IL-4 promoter to positively regulate IL-4. (**A**) Dose dependence of the KLF13 enhancement on IL-4 promoter luciferase activity. (**B**) Potential KLF13 binding sites were confirmed using IL-4 luciferase construct mutated on CACCC sequence. (**C**) Direct binding of KLF13 to the IL-4 promoter was confirmed by ChIP assay. (**D**) KLF13 binds to both the RANTES (positive control) and IL-4 promoters in a gel shift assay. *Lanes 1 and 4*, Biotin-labeled RANTES or IL-4 probe. *Lanes 2 and 5*, Biotin-labeled probe plus mouse KLF13 protein. *Lanes 3 and 6*, Biotin-labeled probe plus mouse KLF13 plus 200-fold excess of unlabeled probe. All results are representative of three independent experiments. $^{*}p < 0.05$.

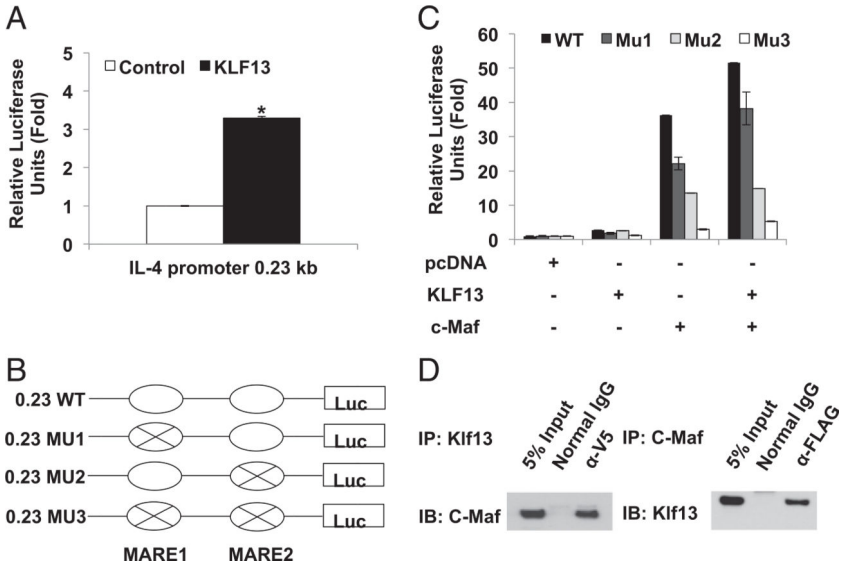

FIGURE 5.

KLF13 synergizes with c-Maf to regulate expression of IL-4. (**A**) KLF13 enhances IL-4 promoter reporter gene activity in the absence of a CACCC binding site. The truncated IL-4 0.23 kb luciferase promoter construct lacking a CACCC site was cotransfected with the KLF13 construct into EL4 cells, and luciferase activity was measured. (**B**) IL-4 promoter luciferase constructs. (**C**) Luciferase activity in EL4 cells transfected as shown. (**D**) KLF-13 coimmunoprecipitates with c-Maf in EL4 cells. Klf13 is V5 tagged, and c-Maf is FLAG tagged. Immunoprecipitation (IP) was performed with anti-V5 antiserum (*left panel*) or anti-FLAG (*right panel*), and coimmunoprecipitated proteins were detected by immunoblotting (IB) with anti-FLAG Ab (c-Maf) or anti-FLAG Ab (Klf13). All results are representative of at least three independent experiments. $*p < 0.05$.

Table I.

Expression of Th2-related cytokines in $CD3^+$ T cells

Gene	RefSeq	Fold Change by Microarray (*p* Value)	Fold Change by PCR
IL-4	NM_021283	−21.84425 (0.0004)	−21.70
IL-5	NM_010558	−21.62425 (0.0019)	−23.12
IL-9	NM_008373	−21.85342 (0.0130)	−22.94
IL-10	NM_010548	−21.68577 (0.0016)	−23.31
IL-13	NM_008355	−21.94515 (0.0022)	−22.80

$CD3^+$ splenocytes from WT and *Klf13*$^{-/-}$ animals were activated for 4 d with anti-CD3 and anti-CD28 Abs. Microarray results were analyzed with Partek Genomics Suite software and confirmed with quantitative RT-PCR. RefSeq, National Center for Biotechnology Information Reference Sequence Database (http://www.ncbi.nlm.nih.gov/refseq/) number.