Stat6 and c-Jun Mediate Th2 Cell-Specific IL-24 Gene Expression

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*J Immunol* published online 25 February 2011
http://www.jimmunol.org/content/early/2011/02/25/jimmunol.1002620
Stat6 and c-Jun Mediate Th2 Cell-Specific IL-24 Gene Expression

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TCR signaling regulates multiple aspects of T cell function by controlling expression of various cytokine genes. IL-24 is a multifunctional cytokine belonging to the IL-10 family. It displays anticancer effects in diverse cancer cells and regulates immunopathology of psoriasis and rheumatoid arthritis. IL-24 also plays an important role in B cell differentiation. Mouse IL-24 gene is selectively expressed in activated Th2 cells upon TCR stimulation. However, the molecular mechanisms by which TCR stimulation induces IL-24 gene expression are still unclear. In this study, to elucidate the mechanism of Th2 cell-specific expression of IL-24, we identified a proximal promoter region (−157/+95bp) that plays critical role in activating the IL-24 gene in Th2 cells. This region has a Th2 cell-specific open chromatin structure along with permissive histone modifications. In vivo binding of Stat6 and AP-1 (c-Jun) to the IL-24 promoter locus in Th2 cells synergistically transactivated the IL-24 promoter. Stat6 and c-Jun proteins were found to physically cooperate with each other and upregulated IL-24 gene transcription. Knockdown of either Stat6 or c-Jun suppressed endogenous IL-24 gene expression in Th2 cells. In summary, TCR stimulation induces IL-24 expression in Th2 cells by the coordinate action of Stat6 and c-Jun transcription factors at the transcriptional level. The Journal of Immunology, 2011, 186: 000–000.

Proper differentiation of naive precursor T cells into effector T cells is necessary to regulate diverse immune responses through coordinated expression of lineage-specific cytokine genes (1, 2). Mutually exclusive patterns of cytokine production in fully committed Th cells (Th1, Th2, or Th17) are precisely regulated by specific transcription factors upon exposure to differentiating stimuli such as high concentrations of TCR stimulation and polarizing cytokines ex vivo (3). Th1 cells help to clear intracellular bacteria and viruses, and their differentiation is driven by transcription factors such as Stat4, Stat1, and T-bet, which lead to production of IL-2, IFN-γ, and lymphotoksin. Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13. Th2 cells promote humoral immunity, and their differentiation requires Stat6 and Gata3. Th17 cells, in contrast, require retinoic acid-related orphan receptor (ROR)γt and express high amounts of IL-17A, IL-17F, and TNF-α, leading to clearance of extracellular bacteria and fungi (4, 5). Several studies have demonstrated the important role of chromatin remodeling and opposing epigenetic regulation mechanisms for transcription of cytokine genes in mammalian cells, including genes that encode IL-4 and IFN-γ (6–9).

IL-24, originally termed as melanoma differentiation-associated gene 7, was identified by subtraction hybridization of cDNA libraries from human melanoma cells (10). It is a member of the IL-10 cytokine family, is located within the IL-10 gene cluster in chromosome 1 along with IL-10, IL-19, and IL-20, and shares both structural and sequence homology with these proteins (11). Forced IL-24 expression using plasmid or adenoviral vectors selectively exhibits tumor-specific proapoptotic, growth inhibitory, and antiangiogenic activities in a broad spectrum of malignant cell lines but has no effect on normal cells (12–14). Although the tumor suppressor role of IL-24 has been well characterized, its immunomodulatory function and its role in inflammatory diseases are still under excessive scrutiny. In the human immune system, certain stimuli promote secretion of IL-24 by PBMCs, preferably monocytes and T and B cells (15, 16). IL-24 is produced not only by activated immune cells but also to a similar extent by non-immune cells such as cultured melanocytes (10), dermal keratinocytes (17), and IL-1–stimulated human colonic subepithelial myofibroblasts (18). IL-24 expression also has been reported in affected joints of rheumatoid arthritis patients (19) and is involved in the immunopathology of psoriasis (17, 20) at the edge of excisional skin wounds (21) and in active lesions from patients who have ulcerative colitis and Crohn’s disease (18). IL-24 inhibits differentiation of germinal center B cells into mature plasma cells and promotes their maturation toward the memory B cell pathway (22). IL-24 also induces secretion of proinflammatory cytokines (IFN-γ, IL-6, and TNF-α) along with lower levels of IL-1, IL-12, and GM-CSF from human PBMCs favoring a Th1-type immune response (15). The secreted IL-24 protein can interact in a paracrine manner with IL-20R1/IL-20R2 and IL-22R1/IL-20R2 receptor complexes, which lead to STAT3 activation (23–25). Although IL-20R2 is expressed in most immune cells, these cells lack the IL-20R1 or IL-22R1 receptors, implying that although...
immune cells produce IL-24, they are not the main targets of IL-24. Several tissues express these receptors and appear to be the main targets of IL-24. These include the tissues from the reproductive and respiratory systems as well as various glands. Under Th1 cytokine conditions, IFN-γ can upregulate IL-22R1 expression in keratinocytes, and a formation of the IL-22R1/IL-20R2 complex promotes the innate, nonspecific immunity of tissues (16, 26). Mice lacking the IL-20R2 show that IL-24 has a largely redundant role in IL-20 and IL-22 in epidermal functions. IL-24 transgenic mice show an increased MCP-1 expression in both keratinocytes and conventional PCR RNA isolation, cDNA synthesis, quantitative real-time PCR, and conventional PCR

Materials and Methods

Mice and cell lines

C57BL/6 mice 6–8 wk of age (SLC, Hamamatsu, Japan) were housed in specific pathogen-free barrier facilities. All animal experiments were performed in accordance to protocols approved by the animal care and use committee of the Gwangju Institute of Science and Technology (Gwangju, Korea). EL4 (murine lymphoma cells) (Korea Cell Line Bank, Seoul National University, Seoul, Korea) and HEK 293T (American Type Culture Collection, Manassas, VA) cells were maintained as described previously (31).

CD4+ T cell isolation, differentiation, and culture

Total RNA was extracted from the stimulated or unstimulated cells using TRIzol Reagent (Molecular Research Center, Cincinnati, OH), according to the manufacturer’s protocol. For reverse transcription, 1 μg total RNA was used, and cDNA was generated using oligo(dT) primer and Improm-II Reverse Transcriptase (Promega, Madison, WI) in a total volume of 20 μl. The mRNA level was determined using 1 μl cDNA by real-time PCR with SYBR premix Ex Taq (Takara, Shiga, Japan) by using a protocol provided by the manufacturer (MJ Research Chromo4, Chromo System, Bio-Rad Laboratories). Mouse hypoxanthine phosphoribosyltransferase (HPRT) primer was used for quantitative RT-PCR or conventional PCR to normalize the amount of cDNA used for each condition. The primer sequences used are as follows: HPRT [5’-TGAATGTTCACGCCTCTAGAG-3’ (forward) and 5’-CTCAGCGGTTGACGCTGCT-3’ (reverse)]; IL-24 [5’-GCCGCAAGGAGAACGAGGAGGCAATCTCA-3’ (forward) and 5’-ATTCTGCAGATCCGATGAGG-3’ (reverse)]; Stat6 [5’-AGTCTATAAAGCCCGCAAACG-3’ (forward) and 5’-GCCACACAGACACAGTGC-3’ (reverse)]; and AP-1 (c-jun)-5’-CTCTTACAGGAGATCCCTC-3’ (forward) and 5’-GTTGCTAAGGATTCTGTTT-3’ (reverse).

Chromatin accessibility by real-time PCR assay

Th1 and Th2 cells (5 × 10^6 cells/sample) stimulated with anti-CD3/28 or PMA/ionomycin for 2 h were pelleted by centrifugation at 500 × g, washed in ice-cold PBS, and then resuspended in 100 μl digestion buffer (110 mM Tris-HCl (pH 7.4), 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, and 1 mM CaCl_2) with or without 5 U micrococcal nuclease (MNase)/ml (Roche, Mannheim, Germany) and were incubated at 37°C for 10 min. Reactions were terminated by adding 20 μl Stop solution [100 mM EDTA and 10 mM EGTA (pH 8.1)]. DNA was isolated using the NuSieve/GlyNPure kit (Intron Biotechnology, Sungnam, Korea) and eluted into 100 μl Tris-EDTA buffer. DNA recovered from MNase samples was checked for fragmentation in 1% agarose gel. RT-PCR analysis was performed in untreated and MNase-treated samples to measure the relative abundance of target regions using the following primer sets: IL-24 promoter [5’-TCATCATCCCTGAAAACTGCT-3’ (forward) and 5’-TCAGTGTAGAGGATCTTCT-3’ (reverse)] and actin promoter [5’-TTCCGAAGTTGCCCTTTATTGCGTCA-3’ (forward) and 5’-AAAGAATCTGCAAGAAGCTTGTG-3’ (reverse)]. Chromatin accessibility values were calculated as a ratio of the undigested sample to the digested samples, and then, the data were plotted as the ratio of accessibility observed in the digested DNA samples for MNase accessibility.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) analysis was carried out as previously described with minor modifications (31, 32). Chromatin from PMA/ionomycin-stimulated primary Th1 and Th2 cells was immunoprecipitated using the Abs to the following: RNA Pol II, Stat6, and c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA); acetyl histone H3 lysine 9/14 (H3AcK9/14), dimethyl histone H3 lysine 4 (H3K4Me2), and histone deacetylase (HDAC) 1 (Upstate Biotechnology, Lake Placid, NY) and rabbit IgG (Sigma-Aldrich). Following reversal of cross-links, the presence of selected DNA sequences was assessed by PCR. As a loading control, PCR was done directly on input DNA purified from chromatin before immunoprecipitation. The primers used were designed with primer3 (http://www.ncbi.nlm.nih.gov/blast) and correspond to regions upstream of the IL-24 promoter region.

Plasmid construction, site-directed mutagenesis, and luciferase reporter assays

The deletion reporter constructs were generated by cloning of the genomic sequences upstream of the first coding exon of the IL-24 gene into the pXP2 reporter vector digested by appropriate restriction enzymes. A reverse primer from the mouse IL-24 gene was used for PCR amplification (+495 primer, 5’-CCCAAGCTTCTGGAGAAGATGTCTC-3’). Different forward primers lying in the 5’-region of the transcription start site of the mouse IL-24 promoter were used to obtain the deletion constructs (−2305 bp, 5’-CAGCTGTGAAGCTGAACTCATGATACGTCTCTAGAAG-3’; −1805 bp, 5’-CCGGTCTGACAAGGCTGAACTCATGATACGTCTCTAGAAG-3’; −1205 bp, 5’-CCGGTCTGACAAGGCTGAACTCATGATACGTCTCTAGAAG-3’; −696 bp, 5’-CAGCTGTGAAGCTGAACTCATGATACGTCTCTAGAAG-3’; −557 bp, 5’-CCGGTCTGACAAGGCTGAACTCATGATACGTCTCTAGAAG-3’; −500 bp, 5’-CCGGTCTGACAAGGCTGAACTCATGATACGTCTCTAGAAG-3’; −450 bp, 5’-CCGGTCTGACAAGGCTGAACTCATGATACGTCTCTAGAAG-3’; −400 bp, 5’-CCGGTCTGACAAGGCTGAACTCATGATACGTCTCTAGAAG-3’; −350 bp, 5’-CCGGTCTGACAAGGCTGAACTCATGATACGTCTCTAGAAG-3’; −300 bp, 5’-CCGGTCTGACAAGGCTGAACTCATGATACGTCTCTAGAAG-3’; −250 bp, 5’-CCGGTCTGACAAGGCTGAACTCATGATACGTCTCTAGAAG-3’; −200 bp, 5’-CCGGTCTGACAAGGCTGAACTCATGATACGTCTCTAGAAG-3’; −150 bp, 5’-CCGGTCTGACAAGGCTGAACTCATGATACGTCTCTAGAAG-3’; −100 bp, 5’-CCGGTCTGACAAGGCTGAACTCATGATACGTCTCTAGAAG-3’; −50 bp, 5’-CCGGTCTGACAAGGCTGAACTCATGATACGTCTCTAGAAG-3’; −25 bp, 5’-CCGGTCTGACAAGGCTGAACTCATGATACGTCTCTAGAAG-3’; −10 bp, 5’-CCGGTCTGACAAGGCTGAACTCATGATACGTCTCTAGAAG-3’; and −5 bp, 5’-CCGGTCTGACAAGGCTGAACTCATGATACGTCTCTAGAAG-3’).
Nuclear extracts and EMSA analysis

Nuclear extracts were prepared from EL-4 or primary Th2 cells stimulated with PMA/ionomycin for 2 h. Cells (2 × 10^6) were washed in ice-cold PBS and suspended in 1 ml lysis buffer (10 mM Tris-HCl, 3 mM CaCl_2, and 2 mM MgCl_2) containing protease inhibitor mixture (Roche) for 10 min on ice. They were vortexed gently and incubated in 1 ml Nonidet P-40 buffer (10 mM Tris-HCl, 3 mM CaCl_2, 2 mM MgCl_2, and 1% Nonidet P-40) for 5 min at 4˚C and centrifuged at 3000 rpm for 10 min at 4˚C. Nuclei were washed twice with 1 ml of serum-free 20 mM HEPES-KOH, 1.5 mM MgCl_2, 10 mM KC1, 0.5 mM DTT, and 0.5 mM PMSF, 100 μl buffer C (20 mM HEPES-KOH, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl_2, 0.2 mM EDTA, 5 mM DTT, 0.5 mM PMSF, and 1% Triton X-100) was added to the pellets, and they were vortexed vigorously at 4˚C for 10 min. Complementary oligonucleotide pairs were annealed in 100 mM NaCl, 10 mM Tris (pH 8), and 0.1 mM EDTA buffer condition by heating to 95˚C for 10 min and cooled slowly to the annealing temperature. The forward primer sequences (consensus sites are underlined, and the mutated residues are in lowercase letters) are as follows: WT IL-24 promoter (Stat6), 5'-AAGTATCCAAGTACCAATGTC; Stat6 mutant, 5'-AAGTATcCAGGGGCTAATGTC-3'; IL-24 Stat6 mutant, 5'-AAGATGCAGGGGGCCTC-3'; WT IL-24 promoter (AP-1), 5'-AAGGCTAATTGGAATGTG-3'; AP-1 consensus, 5'-CTGTTGAGATGGAGCTG-3'; IL-24 AP-1 mutant, 5'-AAGGTTGGAC-3'; IL-24 AP-1 mutant, 5'-AAGGGTAGCATGATG-3'. Double-stranded oligonucleotides were end-labeled with γ-32PATP (PerkinElmer, Wellesley, MA) and T4 polynucleotide kinase (Promega). Labeled oligonucleotides were purified using microspin G-50 column (275330; Amersham Biosciences, Uppsala, Sweden). The nuclear extract (2 μg) was added to binding buffer (10 mM Tris (pH 7.5), 0.5 mM MgCl_2, 80 mM NaCl, 2.5 mM DTT, 4% glycerol, 1 mM 2-ME) along with 0.1 μg/ml poly(dI-dC) and in the presence or absence of the labeled WT or mutant probes and incubated at 4˚C for 30 min. For cold competition, unlabeled WT, consensus, or mutant oligonucleotides in 50-fold molar excesses was added and preincubated for 20 min. For supershift assays, nuclear extracts were preincubated for 30 min with 2 μg anti-Stat6, anti-c-Jun, or control IgG Abs. The samples were separated in 4% nondenaturing polyacrylamide gel containing 0.5% Tris borate-EDTA at 4˚C. Coimmunoprecipitation assay and immunoblotting

HEK293T cells were transfected by using Lipfectamine 2000 (Invitrogen, Carlsbad, CA) with Stat6- and c-Jun-expressing plasmids. Cells were stimulated for 2 h with PMA/ionomycin and then harvested, washed with ice-cold PBS at 48h posttransfection, and lysed in cell lysis buffer (50 mM Tris (pH 8), 0.5% Nonidet P-40, 10% glycerol, 0.1 mM EDTA, 100 mM NaCl, 50 mM NaF, 1 mM Na_2VO_4, 1 mM DTT, and protease inhibitor mixture (Roche)). Primary Th2 cells differentiated in vitro (9) were restimulated and untreated the same way. Cell lysates of in vitro-differentiated Th2 cells were either treated with 100 μg/ml DNase I (Roche) for 20 min at 37°C or 50 μg/ml ethidium bromide (EB; Bio-Rad, Richmond, CA) on ice for 30 min or left untreated. The cell lysates (1 μg) were preclarified by incubation with control Ig for 1 h, followed by the addition of either protein A- or protein G-Sepharose at 4˚C for 2 h. Cleared lysates were immunoprecipitated separately with 2 μg each of Stat6 or c-Jun Abs for 2 h, followed by the addition of 30 μl of either protein A- or protein G-Sepharose for 2 h. The immunoprecipitates were washed in cell lysis buffer four times and separated by SDS-PAGE. The proteins were transferred to nitrocellulose membranes and analyzed by Western Blot with the indicated primary or phosphotyrosine Abs diluted in TBS-T buffer (10 mM Tris (pH 7.5), 150 mM NaCl, and 1% Tween 20) supplemented with milk powder (5%). The blots were visualized using the ECL system (Amersham Biosciences, Buckinghamshire, U.K.). For straight Western blotting, totally differentiated Th1 or Th2 cells were stimulated with PMA/ionomycin for 2 h and were lysed in cell lysis buffer described above. Thirty micrograms of whole-cell lysate or nuclear extract was used for SDS-PAGE, and the Western blot was carried out with the indicated Abs.

Small interfering RNA transfection assay

The predicted small interfering RNAs (siRNAs) for Stat6 (sc-63570), c-Jun (sc-29224), and control siRNA were purchased from Santa Cruz Biotechnology. Fully differentiated Th2 cells were cultured with 60–70% confluency in fully supplemented T cell medium. The cells were then subjected to Nucleofection (Amaxa Biosystems), according to the manufacturer’s protocol. The total amount of transfected plasmids for each sample was normalized by adding the control vector pCMV or pcDNA. After 18 h, cells were stimulated with PMA/ionomycin or anti-CD3/28 as indicated in the figures and harvested, and luciferase activity was assessed by the Dual Luciferase Assay system (Promega). Cotransfection of the pRL-TK vector as an internal control allowed normalization of transfection by Renilla luciferase activity.

Computational analysis of the IL-24 locus

To identify potential regulatory locus, comparative genomic analysis was performed. Genomic sequences spanning the IL-24 gene were analyzed using the web-based alignment software VISTA browser 2.0 (33). Transcription factor binding sites were identified using the rVISTA 2.0 software (34), which uses matrices of the TRANSFAC database (35). Putative recognition sites for regulatory factors also were identified by searching the JASPAR database (36) and verified from the previously reported literature.

Statistical analysis

Data are the mean of SE of at least three independent experiments, unless differently specified in the text. The Student t test was used to determine the significance of the results (significance at *p < 0.05, **p < 0.01, ***p < 0.001). Significance was only indicated when appropriate.

Results

Differential IL-24 gene transcription in Th1 and Th2 cells

To investigate the underlying mechanism of differential IL-24 gene expression in Th cell subsets, we analyzed IL-24 mRNA expression profile in Th1 and Th2 cells differentiated in vitro. CD4+ T cells isolated from spleen and lymph nodes of C57BL/6 mice were either left unstimulated (without) or cultured under Th1 and Th2 differentiation conditions for 6 d as described in Materials and Methods. Cells were then restimulated with PMA/ionomycin or anti-CD3/CD28 for the indicated time points, and IL-24 mRNA expression was analyzed by quantitative RT-PCR (Fig. 1A). The mean fluorescence intensity of IL-24-transfected and nontransfected Th2 cells was >10 fold higher than that of untransfected Th2 cells, even in the absence of stimulation. IL-24 transcript levels in Th2 cells were significantly higher than Th1 cells at all time points tested in the PMA/ionomycin-stimulated cells (Fig. 1A). This increase was pronounced upon 1 h of stimulation in Th2 cells and was maximal at 2 h (>50-fold) of stimulation (Fig. 1A). Similar expression kinetics was observed upon anti-CD3/CD28 stimulation (Supplemental Fig. 1). We also measured IL-24 mRNA expression profiles during Th cell differentiation. Th2 cells began to show a significant increase of IL-24 expression after day 3 in the differentiating culture conditions and progressively increase its expression till day 6, whereas Th1 cells failed to increase IL-24 expression during the period (Fig. 1B). IL-4 and IFN-γ mRNA levels were also analyzed as controls to validate our in vitro differentiation and culture system (Fig. 1C).

Identification of Th2 cell-specific functional IL-24 promoter

Compared with Th1 cells, Th2 cells express much higher levels of IL-24 gene (Fig. 1), suggesting that Th2 cells have unique transcription machinery to activate the IL-24 promoter. To identify
and characterize the potential regulatory elements in the 5′-flanking region of the mouse IL-24 gene, we performed comparative genomic sequence analysis. The DNA sequences of IL-24 genomic loci of the murine, rat, and human were compared with highly conserved noncoding sequences as potential regulatory elements (Fig. 3A). A series of deletion constructs containing the 5′-end of the IL-24 gene were cloned upstream to the luciferase reporter to delineate the minimum region with the highest transactivity. The longest construct was designed from position 2305 to +95 (2.4 kb construct). The genomic position and the size of the constructs are shown in Fig. 2A. Reporter assays in EL-4 T cells showed stimulation-dependent transactivity of all the constructs. On the basis of the reporter assay, the proximal promoter region was identified to be located in the 2157/+95 region (252 bp construct) that showed maximal activity upon TCR stimulation (Fig. 2A). Although the basal activity of this promoter region was

**FIGURE 1.** Th2 cell-specific expression of IL-24. A, CD4+ T cells from C57BL/6 were cultured under Th1- or Th2-skewing conditions as described in Materials and Methods. Total RNA was isolated from the 6-d differentated Th1 and Th2 cells, which were either left unstimulated [without (w/o)] or restimulated with PMA/ionomycin (PMA/Iono) for the indicated time points and subjected to real-time PCR (RT-PCR) analysis. Mouse HPRT was used as a control. B, Upregulation of IL-24 mRNA during in vitro differentiation of Th2 cells. Cells were cultured under Th1 or Th2 conditions for indicated time periods and restimulated with PMA/ionomycin for 2 h, and then, real-time PCR analysis was performed as described above. Negative images of EB-stained gels depict IL-24 gene expression at days 0, 3, and 6 in both Th1 and Th2 cells. C, The expression levels of IL-24, IFN-γ, and IL-4 from the in vitro-differentiated Th1 and Th2 cells were analyzed by RT-PCR. The data shown in A and B are expressed as mean ± SEM, n = 3. *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 2.** Identification of promoter region of the mouse IL-24 gene. A, EL4 T cells were transfected with the control pXPG vector (mock) and the IL-24 luciferase reporter constructs. Schematic representation of the genomic position and size of the deletion constructs of the 5′-region of the mouse IL-24 gene are shown. Twenty-hour posttransfection cells were left unstimulated [without (w/o)] or stimulated with PMA/ionomycin for 4 h and then harvested for assay. Luciferase activity is expressed relative to the expression of a cotransfected Renilla luciferase plasmid (pRL-TK) as a control for transfection efficiency. Luciferase units are expressed as a fold difference relative to the control value. B, CD4+ T cells from C57BL/6 were cultured under Th1- or Th2-skewing conditions for 6 d and transfected with the control pXPG vector or the IL-24 luciferase reporter constructs as indicated. After transfection, cells were stimulated with anti-CD3/28 prior to harvest. C, The IL-24 promoter (252 bp) reporter construct was cloned in the reverse orientation (OR) and transfected in EL4 T cells, as described above. The graphs in B and C represent mean ± SEM, n = 3. *p < 0.05, **p < 0.01. D, NIH3T3 and M12 cells were transfected with the control pXPG vector or the IL-24 luciferase reporter construct (252 bp), stimulated with PMA/ionomycin, and harvested for luciferase assay. Luciferase activity is expressed relative to the expression of a cotransfected Renilla luciferase plasmid (pRL-TK) as a control for transfection efficiency. Luciferase units are expressed as the fold difference relative to the control value.
low (5-fold) compared with empty vector, it was highly induced upon PMA/ionomycin stimulation (>40-fold) in EL4 cells (Fig. 2A). A further deletion of 107 bp (−50/+95 region; 145 bp construct) showed almost no significant activity both in EL4 cells (Fig. 2A) and primary T cells (Fig. 2B). We also tested promoter activity of the diverse reporter constructs in primary Th1 and Th2 cells. All the constructs showed higher transactivity upon anti-CD3/CD28 stimulation only in Th2 cells but not in Th1 cells (Fig. 2B). The 252 bp construct (−157/+95) showed maximal activity (>4-fold) upon stimulation in Th2 cells compared with Th1 cells (Fig. 2B). Similar results were obtained upon PMA/ionomycin stimulation in the Th cells (Supplemental Fig. 2). The activity of this region was orientation dependent because an inverted IL-24 promoter failed to transactivate upon stimulation (Fig. 2C). Moreover, activity of this proximal promoter region was found to be T cell specific, because it was nonfunctional in B cell lymphoma (M12) or fibroblast (NIH3T3) cells (Fig. 2D). This proximal promoter region lies within the 157-bp upstream region of the transcription start site and is highly conserved (>75%) in humans and mice (Fig. 3A, boxed region). Sequence analysis of this region showed the presence of a TATA box and binding sites for several transcription factors (Fig. 3B). The transcription start site is indicated (arrow) and is designated as +1 (Fig. 3B). The above results suggest that the 252-bp region has T cell lineage-specific promoter activity and is especially functional in Th2 cell subset.

Permissive IL-24 chromatin structure in the Th2 cells

One of the major changes associated with active gene transcription is chromatin remodeling across the gene promoter region, which can be measured by altered accessibility of the chromatin across specific regions using MNase or restriction enzymes (37, 38). Because IL-24 is specifically expressed in Th2 cells upon stimulation, we analyzed whether the chromatin architecture in the area of the proximal promoter is more accessible to the transcriptional machinery in Th2 cells than in Th1 cells. To gain insight into the localized chromatin changes, we examined the accessibility of the IL-24 promoter locus spanning the 252-bp region to MNase by a PCR-based nuclease hypersensitivity assay (39), (Fig. 4A, Supplemental Fig. 3). Nuclear isolated from Th1 or Th2 cells unstimulated or restimulated (both with anti-CD3/28 and PMA/ionomycin) were exposed to MNase, which cuts accessible linker DNA between free nucleosomes. In Th1 cells, the genomic DNA spanning the 252-bp region was less accessible to MNase digestion compared with mouse actin promoter locus, and this accessibility was unaffected upon stimulation, indicating that the IL-24 locus in Th1 cells exists in a compact and condensed chromatin structure. However, the accessibility of the entire probe region to MNase was higher in unstimulated Th2 cells than that of Th1 cells and was further increased upon stimulation. The accessibility of the IL-24 promoter locus was increased by about two times in stimulated Th2 cells than in the stimulated Th1 cells (Fig. 4A, Supplemental Fig. 3), suggesting that the chromatin encompassing the IL-24 promoter region of Th2 cells is decondensed and wrapped loosely around free nucleosomes. The mouse actin promoter locus was similarly accessible in both stimulated and unstimulated Th1 and Th2 cells. Because PMA/ionomycin or anti-CD3/CD28 stimulation showed similar expression profile and MNase sensitivity at the IL-24 locus, we used 2-h PMA/ionomycin-stimulated Th1 and Th2 cells for all further experiments. Acetyl histone H3 lysine9/14 (H3AcK9/14) and histone H3 lysine 4 methylation (H3K4Me2) are typical features associated with active transcription regulating the appropriate tissue-specific and context-dependent induction of various genes (40, 41). Therefore, we analyzed the recruitment of H3AcK9/14 and H3K4Me2 on the IL-24 promoter in the Th1 and Th2 cells upon stimulation by ChIP assay (Fig. 4B). In stimulated Th1 cells, the nucleosomes positioned across the IL-24 proximal promoter region exhibited little or no H3AcK9/14 and H3K4Me2, whereas strong signals were observed in Th2 cells (Fig. 4B). The same target regions were amplified efficiently from nonimmunoprecipitated (input) genomic DNA derived from the same cell extract. Higher recruitment of RNA Pol II, preferably in the Th2

File: FIGURE 3. Comparative analysis of the mouse and human IL-24 gene loci. A, rVISTA2.0 analysis of the mouse and human IL-24 loci is shown. The mouse genomic sequence is used as the base sequence on the x-axis. The boxed region indicates the putative minimal promoter region of the IL-24 gene critical for its Th2 cell specificity. B, Comparison of the genomic sequence of murine and human IL-24 promoter regions. The transcription start site is indicated (arrow) and is designated as +1. Potential TATA box and transcription factor binding sites that meet the most stringent requirements, using rVISTA 2.0 and TRANSFAC database analysis, are indicated and boxed.
cells, further confirmed the Th2 cell specificity of IL-24 gene expression. As control experiments, the promoters of IL-4 and IFN-γ were analyzed in these same cells (Fig. 4B). We also measured in vivo binding of HDAC1 in the IL-24 promoter region to further confirm the association of silencing complex in Th1 cells. Indeed, HDAC1 was more strongly associated with the IL-24 promoter in the Th1 cells than in the Th2 cells. The mouse actin promoter did not recruit HDAC1 in either Th1 or Th2 cells (Fig. 4C), which serves as an experimental control. We also tested the effect of treatment of TSA, an HDAC inhibitor, on IL-24 expression in Th1 cells. Indeed, TSA treatment significantly increased the IL-24 expression in Th1 cells in a dose-dependent manner (Fig. 4D), although the expression level of IL-24 was still low (~20%) compared with that of Th2 cells. Treatment of TSA did not show any significant changes in IL-24 expression in Th2 cells (Supplemental Fig. 4). These results indicate that the IL-24 promoter in Th2 cells has accessible chromatin structure, whereas it remains inaccessible in Th1 cells possibly through an association of silencing machinery.

In vitro and in vivo binding of Stat6 and c-Jun to the IL-24 promoter locus

On the basis of the finding that the IL-24 promoter showed Th2-sp. act., whereas Th1 cells failed to activate it (Fig. 2B), we sought to identify the transcription factors that could bind to the IL-24 gene promoter and activate its transcription. Sequence analysis of the IL-24 proximal promoter region (−157/95) showed the presence of a highly conserved Stat6 binding site adjacent to an AP-1 binding site (Fig. 3B). Because IL-24 is specifically produced by Th2 cells, and Stat6-deficient mice fail to express IL-24 even under Th2 culture conditions (29), we investigated whether Stat6 plays a direct role in controlling IL-24 gene expression in Th2 cells. Physical association of Stat6 to the IL-24 promoter region was analyzed in vitro by gel shift assays using nuclear extracts prepared from PMA/ionomycin-stimulated EL4 T cells and primary Th2 cells (Fig. 5A–C). Indeed, Stat6 binding to the IL-24 promoter sequence was observed (Fig. 5A left panel, lane 1, upper band marked by arrow). Presence of excess competitor oligonucleotides, both of IL-24 promoter probe (WT) and Stat6 consensus probe (Fig. 5A, left panel, lanes 2, 4), diminished the complex formation. However, oligonucleotides containing a mutant Stat6 site did not show complex formation (Fig. 5A, left panel, lane 7) and failed to decrease the level of competition for Stat6 binding (Fig. 5A, left panel, lanes 5, 6). Addition of anti-Stat6 Ab showed a shift in the protein/oligonucleotide complex, whereas isotype IgG did not (Fig. 5B, left panel), which further confirms the specificity of Stat6 binding. Next, we tested whether AP-1 also plays a direct role in controlling IL-24 gene expression in Th2 cells. Of the AP-1 family, Jun proteins are preferably expressed in Th2 cells. Of the AP-1 family, Jun proteins are preferably expressed in Th2 cells. Of the AP-1 family, Jun proteins are preferably expressed in Th2 cells. Among them, Jun B is shown to be accumulated in activated Th2 cells, whereas c-Jun and Jun D are expressed similarly in activated Th1 and Th2 cells (43). Although the binding of c-Jun to the human IL-24 promoter locus has been reported in other cells (18, 30), its role in regulating IL-24 ex-
pression in T cells has not been elucidated. Because the AP-1 binding site is highly conserved in humans and mice, to check whether c-Jun could bind to the IL-24 promoter in T cells, we performed EMSA in both EL-4 and Th2 cells (Fig. 5A–C). c-Jun binds to the IL-24 promoter probe (Fig. 5A, right panel, lane 1). Excess competitor oligonucleotides (IL-24 promoter-WT or c-Jun consensus) inhibited c-Jun binding to the probe (Fig. 5A, right panel, lanes 2, 4), whereas mutant oligonucleotides failed to inhibit the complex formation (Fig. 5A, right panel, lane 6). To further confirm the complex c-Jun/DNA complex formation, c-Jun Ab was added, and shift in the complex was measured. Although c-Jun Ab treatment did not induce a shift, it decreased the intensity of the complex (Fig. 5B, right panel, lane 3). As a control, we confirmed that isotype IgG showed no such effect (Fig. 5B, right panel, lane 2). We saw similar Stat6 and c-Jun binding in primary Th2 cells as in EL-4 cells (Fig. 5C). Addition of c-Jun or Stat6 Abs showed a pronounced decrease in the respective complex formations. To further check whether these two factors also bind to IL-24 promoter region in vivo, ChIP assay was performed in both Th1 and Th2 cells stimulated with PMA/ionomycin for 2 h (Fig. 5D, 5E). Control normal rabbit serum did not immunoprecipitate any factor bound to the IL-24 promoter. Binding of both endogenous Stat6 and c-Jun to the IL-24 promoter locus was highly enriched in Th2 cells but not in Th1 cells. As positive controls, we confirmed c-Jun and Stat6 binding to the previously reported IL-10 (CNS3) and Th2 (RHS7) loci, respectively (42, 44). The above result indicates that Stat6 and c-Jun sites are physically associated with an IL-24 promoter region in a Th2-specific manner.

**Functional synergy of Stat6 and c-Jun in transactivating the IL-24 promoter**

Association of both Stat6 and c-Jun with proximal IL-24 promoter locus in vitro and in vivo suggests that these factors could be involved in the transcriptional activation of IL-24 gene. To test this hypothesis, we performed a luciferase reporter assay in the presence or absence of those factors. EL-4 T cells were transiently cotransfected with an IL-24 proximal promoter (252 bp, −157/ +95) vector and increasing amounts of either Stat6 or c-Jun expression vectors or empty control vectors (Fig. 6A). IL-24 transcriptional activity was induced by c-Jun expression in a dose-dependent manner and showed maximal transactivation with the highest concentration of c-Jun used. Stat6 induced IL-24 promoter activity in the same way as that of c-Jun (Fig. 6A). To analyze whether Stat6 and c-Jun exert a coordinate action on IL-24 transcriptional activity, we cotransfected both factors, at the lowest effective concentration (0.2 μg), and measured their effect on IL-24 promoter. Cotransfection of Stat6 and c-Jun synergistically increased (>6-fold) luciferase activity (Fig. 6B) compared with promoter alone, suggesting a cooperative role of these factors in regulating IL-24 promoter activity. Mutation of the Stat6 binding site in the IL-24 promoter element decreased the endogenous activity of the IL-24 promoter by 30% upon stimulation (Fig. 6C). The effect was more pronounced upon c-Jun binding site mutation where a 60% downregulation of the promoter activity was seen. Upon disruption of both binding sites, the IL-24 promoter lost ~80% of its activity upon stimulation (Fig. 6C), confirming the involvement of both Stat6 and c-Jun in TCR-induced IL-24 promoter activity. To test whether Th2-specific IL-24 expression is
linked with their exclusive expression, we compared protein levels of Stat6 and c-Jun between Th1 and Th2 cells. No significant difference was observed in the total cell lysates. However, Stat6 levels were significantly higher in the nucleus of Th2 cells than in Th1 cells, although c-Jun levels were similar (Fig. 6D). This result suggests that an increased nuclear level of Stat6 in Th2 cells in cooperation with c-Jun could mediate upregulation of IL-24 mRNA in a Th2 cell-specific way.

Physical interaction between Stat6 and c-Jun

To check whether the functional synergy between Stat6 and c-Jun observed in the IL-24 reporter assays (Fig. 6B) is linked with their physical association, HEK293T cells were cotransfected with expression vectors for Stat6 and c-Jun and stimulated for 2 h with PMA/ionomycin prior to harvest. The whole-cell lysates from stimulated cells were immunoprecipitated with either Stat6 or c-Jun or isotype control Abs and then resolved on a SDS-PAGE. Immunoblotting with anti-c-Jun or anti-Stat6 revealed that Stat6 and c-Jun proteins physically interact (Fig. 7A). To further detect a direct interaction between endogenous Stat6 and c-Jun proteins, whole-cell lysates were prepared from Th2 cells stimulated for 2 h with PMA/ionomycin, and immunoblotting was performed in the same way as in HEK293T cells. Indeed, a physical interaction also was observed between the endogenous Stat6 and c-Jun proteins in Th2 cells (Fig. 7B). Because both Stat6 and c-Jun are transcription factors and are usually associated with the chromatin in cells, we checked whether the interaction between Stat6 and c-Jun was mediated by the binding of both proteins to the DNA. Interestingly, pretreatment of cell lysates with DNase I or EB prior to coimmunoprecipitation did not affect the interaction of Stat6 and c-Jun (Fig. 7C), indicating that the observed interaction is probably DNA independent.

Stat6 and c-Jun are essential for IL-24 gene expression

To further test the functional involvement of Stat6 and c-Jun in IL-24 production, EL-4 T cells were overexpressed with Stat6 and c-Jun expression plasmids, and then, the IL-24 transcript level was measured by quantitative RT-PCR. Transfection of either Stat6 or c-Jun increased IL-24 expression by 2- or 6-fold, respectively, compared with control cells. Overexpression of both Stat6 and c-Jun synergistically increased the IL-24 expression level by 12-fold compared with the control cells (Fig. 8A). Next, we also analyzed whether Stat6 and c-Jun inhibition could affect endogenous IL-24 production in Th2 cells. We used siRNAs to decrease the endogenous Stat6 and c-Jun expression in Th2 cells, which express high levels of the IL-24 transcript. Indeed, transfection of Stat6 or c-Jun siRNA diminished the expression levels of these transcription factors in Th2 cells compared with cells transfected with control siRNA (Fig. 8B, Supplemental Fig. 5A, 5B). Expression of HPRT remained unaltered in the control siRNA or Stat6- and c-Jun siRNA-transfected cells (Fig. 8B). The inhibition of Stat6 or c-Jun expression decreased IL-24 mRNA production by 60 or 80%, respectively, as analyzed by quantitative RT-PCR (Fig. 8C). The expression of IL-4 and IL-10, which are target genes of Stat6 and c-Jun, respectively, were also downregulated by transfection of relevant siRNAs (Supplemental Fig. 5C, 5D). The c-Jun levels were intact in Stat6 siRNA-transfected cells and vice versa, meaning that the knockdown was target gene specific (Supplemental Fig. 5A, 5B). When Th2 cells were cotransfected with both Stat6 and c-Jun siRNA, IL-24 expression was further diminished by 90% (Fig. 8C). Altogether, the above results corroborate that Stat6 and c-Jun play an important role in regulating endogenous IL-24 expression in response to TCR stimulation in Th2 cells.

FIGURE 6. Synergistic trans activation of IL-24 promoter by Stat6 and c-Jun. A, EL4 T cells were transfected with luciferase reporter constructs containing the IL-24 promoter (252 bp) region along with empty control plasmid (Mock) or expression plasmids encoding Stat6 or c-Jun at various concentrations as indicated. Twenty-hour posttransfection cells were stimulated with PMA/ionomycin for 4 h and harvested for luciferase assay. B, EL4 T cells were transfected with luciferase reporter constructs containing the IL-24 promoter (252 bp) region along with empty control plasmid or expression plasmids encoding Stat6 or c-Jun or both, as indicated, stimulated with PMA/ionomycin for 4 h and harvested for luciferase assay. C, EL4 T cells were transfected with the control pXPG vector, IL-24 promoter (252 bp) reporter vector, or mutant IL-24 promoter reporter constructs with Stat6 (S) or c-Jun (A) binding site mutations (X, mutated site). After transfection, cells were stimulated with PMA/ionomycin and harvested for luciferase assay. The graphs in A–C represents mean ± SEM, n = 3. *p < 0.05, **p < 0.01, ***p < 0.001. D, Stat6 and c-Jun levels in total cell lysates and nuclear extracts of Th1 and Th2 cells stimulated with PMA/ionomycin were assayed by Western blotting using anti-Stat6 and anti–c-Jun Abs. Anti-GAPDH and anti-Lamin B were used as loading controls. Data are representative of three independent experiments.
Discussion

The main purpose of this study was to elucidate the molecular regulation mechanism of the mouse IL-24 gene in CD4+ Th cells. We identified a functional proximal promoter region (~157/+95 bp region) in the 5′-end of the IL-24 gene that has Th2 cell sp. act. We demonstrated that two important families of transcription factors, Stat6 and AP-1 (c-Jun), cooperatively regulate transcription of IL-24 gene in Th2 cells. Preferential in vivo binding of Stat6 and c-Jun to the IL-24 promoter in Th2 cells increases transcriptional activity of the IL-24 promoter. Overexpression of Stat6 and c-Jun upregulated IL-24 expression, whereas the knockdown of Stat6 and c-Jun by relevant siRNAs significantly downregulated endogenous IL-24 synthesis in Th2 cells. Our results also constitute, to our knowledge, the first report of chromatin-level structural differences within the IL-24 gene promoter in differentiated Th1 and Th2 cells and suggest that the effect of TCR signaling on IL-24 transcription seems to require the combined action of Stat6/c-Jun, as well as active chromatin remodeling and epigenetic modifications in the IL-24 promoter locus.

Mouse IL-24 gene expression is specifically upregulated in Th2 cells upon anti-CD3 (29) (Supplemental Fig. 1) or PMA/ionomycin stimulation (Fig. 1). By performing bioinformatics and deletion analysis of the 5′-prime region of the mouse IL-24 genomic locus, we have identified a distinct 252-bp proximal promoter region from −157 bp to +95 bp, which is mainly responsible for TCR-induced Th2-specific IL-24 gene activation. This IL-24 promoter region had orientation-dependent activity and showed Th2 cell-specific transactivation, supporting the possible role of cell-specific factors in regulating IL-24 expression (Fig. 2). The reduced promoter activity with additional upstream sequence suggests that control of the IL-24 promoter is complex and is dependent on positive, as well as negative, regulatory mechanisms (Fig. 2A). According to our results, although the 1.9- and 2.4-kb reporter constructs showed lower levels of luciferase activity than the 252 bp in EL-4 T cells, their activity was much higher in Th2 cells than in Th1 cells (Fig. 2B, Supplemental Fig. 2). The transactivity of the 5′-upstream IL-24 promoter constructs were higher and well maintained in the stimulated Th2 cells, confirming the fact that the Th2 cells have a more permissive chromatin environment that leads to the Th2 cell-specific expression of the IL-24 gene. In fact, the 1.9- and 2.4-kb constructs also showed higher luciferase activity in Th2 cells than in Th1 cells, although their endogenous activity is comparatively lower than the 252-bp promoter.
construct (Fig. 2B, Supplemental Fig. 2). However, a possible negative regulatory locus may exist between the 1.3- and 2.4-kb region of the IL-24 gene. Currently, we are trying to identify repressor elements binding to the above (1.3–2.4 kb) locus.

IL-24 expression kinetics reveals that an increase in the transcriptional competence of the IL-24 locus starts at day 2 after the initiation of Th2 differentiation and accelerates thereafter (Fig. 1B). A key control point in inducible gene expression involves modified histone and chromatin structure at the promoters of the genes that are actively transcribed and may represent a mechanism by which TCR signaling induces effective IL-24 expression. Increased intrinsic accessibility to MNase (Fig. 4A, Supplemental Fig. 3) was observed in the promoter locus spanning the Stat6 and c-Jun binding sites in stimulated Th2 cells along with increased amount of acetylated histone (H3AcK9/14), H3K4Me2, and RNA Pol II recruitment than in the Th1 cells (Fig. 4B), demonstrating that these sites are in an active chromatin conformation in Th2 cells. The accessibility of the IL-24 promoter region in unstimulated Th2 cells was also much higher than that of unstimulated Th1 cells (Fig. 4A, Supplemental Fig. 3), thus explaining the 10-fold higher expression of IL-24 mRNA in unstimulated Th2 cells than that of the Th1 cells (Fig. 1A). However, in committed Th1 cells, the mechanism of IL-24 gene silencing may be attributed to the repressive histone modifications in its promoter. The observation that the promoter of the IL-24 gene in Th1 cells had decreased accessibility even after stimulation and had a closed chromatin structure was further confirmed by the upregulation of IL-24 transcription in TSA (HDAC inhibitor)-treated Th1 cells (Fig. 4D). Whether or not the increased HDAC1 recruitment to the IL-24 proximal promoter locus (Fig. 4C) is mediated by other repressive factor(s) prebound to the IL-24 genomic locus specifically in the Th1 cells awaits further analysis. Thus, chromatin remodeling may contribute to the cell-specific expression of IL-24, controlling the access of appropriate key transcription factors and the transcriptional machinery to the promoter.

Transactivation of the human IL-24 promoter by c-Jun and CEBP-β in human melanoma cells has been reported previously (30). The highly conserved binding site of c-Jun on the IL-24 promoter suggests that c-Jun also may contribute to the Th2-specific expression of IL-24. Although AP-1 proteins have been implicated as important regulators of Th2 cytokine expression, and a higher expression of JunB is reported in Th2 cells (43), we could not see transactivation of the IL-24 promoter upon overexpression of Jun B (data not shown). In addition, there is evidence of subset-restricted binding of c-Jun to cytokine regulatory regions as a result of chromatin remodeling during Th cell differentiation, wherein c-Jun binds to the IL-10 CNS3 region in Th2 cells (42). Hence, although c-Jun is nonselectively induced in both Th1 and Th2 cells, it can still operate in a subset-specific manner by cooperating with other transcription factors with restricted nuclear expression or function, such as Stat6, which has a Th2 cell-specific nuclear expression and function (Fig. 6D). Stat6, in contrast, plays a main role in mediating biological functions of cytokines such as IL-4 and IL-13, which are related to the Th2 branch of the immune system (45, 46). Because mouse IL-24 is reported to be an IL-4–induced secreted protein (FISP), presence of a conserved Stat6 binding site in its proximal promoter compelled us to investigate whether it regulates IL-24 expression directly by binding to the promoter locus. Reporter gene and ChIP assays demonstrated that Stat6 and c-Jun directly bind to and activate the IL-24 promoter (Figs. 5, 6). Interestingly, Stat6 and c-Jun bind to the IL-24 promoter in Th2 cells and not in Th1 cells (Fig. 5D, 5E); therefore, binding of these factors correlates with the high levels of IL-24 produced by Th2 cells. The specificity of the binding sites and the factors binding there were shown by competition studies by using consensus sequences of the transcription factor binding sites and Abs to both Stat6 and c-Jun in EMSA experiments (Fig. 5A–C). Mutations in the Stat6 or c-Jun binding sites prevented formation of the protein/DNA complexes (Fig. 5A) and decreased the promoter activity (Fig. 5C). EMSA with the AP-1 binding site containing probe usually showed a single major complex in both stimulated EL-4 and Th2 cells (Fig. 5A, 5B). This complex formation was decreased by c-Jun consensus sequence, and its intensity was also decreased upon incubating the nuclear extract with anti–c-Jun Ab. The decrease was more pronounced in the primary Th2 cell extracts. Because EMSA shows in vitro binding of DNA and protein, we performed this experiment in only Th2 cells as both Th1 and Th2 cells express similar levels of c-Jun protein and Stat6 protein (Fig. 6D). c-Jun is associated with endogenous IL-24 expression in mouse T cells as evident from our results, so we suggest a predominant role of c-Jun in activating the IL-24 gene. In addition to Stat6 and c-Jun, other transcription factors also may play a role in activation of IL-24 gene expression in Th2 cells. Although bioinformatic analysis predicted the presence of NFAT and ETS binding sites in this proximal promoter region, we were unable to detect activation of the IL-24 promoter in EL4 T cells by TCR stimulation upon cotransfection of NFAT and ETS family factors (data not shown). In addition, we were also unable to find any abnormality in IL-24 expression in either NFAT1– or ETS1-deficient Th2 cells (data not shown). The requirement of additional cell type-specific factors was not obvious in the reporter assays, because the presence of further upstream regions did not enhance transcription (Fig. 2B, 2D).

Functional cooperation between Stat6 and c-Jun has been demonstrated by reporter gene assays whereby Stat6 and c-Jun bind at adjacent sites on the mouse IL-24 gene promoter (Fig. 3B) and enhance gene transcription (Fig. 6B), possibly through a physical interaction between them (Fig. 7). Although functional synergy between Stat6 and c-Jun has been observed previously in activating the mouse germline IgE promoter (47), but to our knowledge, this is the first report demonstrating a physical interaction between Stat6 and c-Jun in CD4+ Th cells along with functional synergy in activating the mouse IL-24 promoter (Fig. 7A, 7B). Furthermore, this interaction is independent of their DNA-binding abilities (Fig. 7C). It is plausible that the cooperation between Stat6 and c-Jun is essential for promoting and strengthening expression of other genes upon TCR stimulation. Interestingly, a 107-bp deletion between –157 and –50 bp, containing the conserved Stat6 and c-Jun binding sites (145 bp construct), caused a significant decrease (~80–90%) of TCR-induced IL-24 promoter activity (Fig. 2A, 2B). This result suggests that this proximal region may act as an enhancer region necessary for the optimal expression of the IL-24 gene by TCR signaling and that Stat6 and c-Jun binding to the IL-24 promoter is absolutely necessary for the response elicited. The dependence of IL-24 gene induction on Stat6 and c-Jun was further confirmed by overexpression and knockdown studies. Forced expression of Stat6 and c-Jun augmented endogenous IL-24 expression in EL-4 cells, whereas siRNA-mediated knockdown of those factors efficiently decreased IL-24 levels in Th2 cells (Fig. 8). These findings are further substantiated by the fact that Stat6-deficient T cells have a defect in IL-24 expression and dominant-negative c-Jun abrogates human IL-24 expression (29, 30). However, further studies are necessary to delineate the role of Stat6 and C-Jun in IL-24 gene regulation in human CD4+ T cells. IL-24 is a potent tumor suppressor cytokine with robust anticancer properties and is an excellent target for gene therapy for cancer (48). Its role in various autoimmune
diseases also has been studied extensively (49). Although tumor suppressor activity of IL-24 is well defined, the role of endogenous IL-24 produced by Th2 cells in autoimmune diseases or in cancer is still unclear. Tumor-infiltrated Th2 cells could act as a stable natural source of IL-24 mediating apoptotic processes or antitumor immune responses. c-Jun is already known to positively regulate IL-24 expression in human melanoma cells (30). Stat6 and c-Jun expression levels often correlate with tumor growth and survival (50–52). Increased Stat6 and c-Jun expression during cancer progression could also promote IL-24 expression in cancer cells, leading to IL-24-mediated antitumor responses. Th2 cell-specific inhibition of IL-24 or functional studies in IL-24-deficient mice are needed to comprehensively address the role of endogenous IL-24 in antitumor immunity, which may lead to novel ways to manipulate IL-24 levels for therapeutic benefits.

In summary, our work constitutes, to our knowledge, the first report demonstrating the transcriptional regulation mechanism of IL-24 gene expression induced by TCR signaling specifically in Th2 cells. IL-24 transcriptional regulation is the result of the coordinated recruitment of the transcription factors Stat6 and c-Jun on the IL-24 proximal promoter in which the cooperation between Stat6 and c-Jun is essential for promoting “ectopic” expression of IL-24 gene in Th2 cells.

Disclosures
The authors have no financial conflicts of interest.

References


