Crucial Role for Nuclear Factor of Activated T Cells in T Cell Receptor-mediated Regulation of Human Interleukin-17*

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The biological activities of the inflammatory cytokine interleukin (IL)-17 have been widely studied. However, comparatively little is known about how IL-17 expression is controlled. Here, we examined the basis for transcriptional regulation of the human IL-17 gene. IL-17 secretion was induced in peripheral blood mononuclear cells following anti-CD3 cross-linking to activate the T cell receptor (TCR), and costimulatory signaling through CD28 strongly enhanced CD3-induced IL-17 production. To define cis-acting elements important for IL-17 gene regulation, we cloned 1.25 kb of genomic sequence upstream of the transcriptional start site. This putative promoter was active in Jurkat T cells following CD3 and CD28 cross-linking, and its activity was inhibited by cyclosporin A and MAPK inhibitors. The promoter was also active in Hut102 T cells, which we have shown to secrete IL-17 constitutively. Overexpression of nuclear factor of activated T cells (NFAT) or Ras enhanced IL-17 promoter activity, and studies in Jurkat lines deficient in specific TCR signaling pathways provided supporting evidence for a role for NFAT. To delineate the IL-17 minimal promoter, we created a series of 5′ truncations and identified a region between −232 and −159 that was sufficient for inducible promoter activity. Interestingly, two NFAT sites were located within this region, which bound to NFATc1 and NFATc2 in nuclear extracts from Hut102 and Jurkat cells. Moreover, mutations of these sites dramatically reduced both specific DNA binding and reporter gene activity, and chromatin immunoprecipitation assays showed occupancy of NFAT at this region in vivo. Together, these data show that NFAT is the crucial sensor of TCR signaling in the IL-17 promoter.

The gene encoding interleukin (IL)-171 was first isolated from an activated rodent T-cell hybridoma, and an emerging family that contains at least six cytokines and five receptors has now been recognized (reviewed in Refs. 1 and 2). Interestingly, both IL-17 and its receptor are distinct in sequence from previously described cytokine/receptor families, although they are quite conserved among mammals. IL-17 has been classified as a key proinflammatory mediator based on its ability to induce a variety of inflammatory effectors (reviewed in Refs. 1 and 3). This cytokine is found at elevated levels in many inflammatory conditions in humans, particularly rheumatoid arthritis and lung airway infections (reviewed in Refs. 4–7), but in the IL-17R also show an impaired ability to recover from Klebsiella pneumoniae infections (13). Similarly, IL-17-deficient mice exhibit reduced airway hypersensitivity responses as well as other immune impairments (14, 15). Thus, it is clear that IL-17 plays a nonredundant role in controlling inflammation in a variety of settings.

Compared with the extensive studies of the biological activities of IL-17, considerably less is known about regulation of IL-17 expression. In humans, IL-17 mRNA is largely found in activated CD4+ memory T cells (16–18), although CD8+ T cells also secrete IL-17 at considerably lower levels (19, 20). In mice, CD4+ and CD8+ T cells produce similar levels of IL-17 upon cross-linking of the T cell receptor (TCR) (20). Although some reports indicate that IL-17 is made by Th0 and Th1 cells, other reports implicate Th2 cells (21, 22). In contrast to the well described cytokine IL-2, CD28 costimulation only mildly enhances IL-17 production in mouse primary cells.2 Under in vitro conditions, the mitogen phorbol 12-myristate 13-acetate (PMA) and the cytokine IL-15 have been shown to stimulate T cells to produce IL-17 (23). Furthermore, data in mice show that lipopolysaccharide, acting through Toll-like receptor-4, stimulates dendritic cells to secrete IL-23, which in turn triggers IL-17 production by T cells (20, 24). However, in IL-23-deficient mice, antigen-presenting cells exhibit only a partially diminished capacity to trigger IL-17 production by T cells, indicating that IL-23 is not the only route to IL-17 production (25). Consistent with this, we recently found that neither dendritic cells nor IL-23 were necessary for promoting short term production of IL-17, although they can enhance its expression ~2-fold.2 Pharmacological inhibitor studies have suggested that TCR-stimulated expression of IL-17 is calcineurin- and cAMP-dependent (23). However, the precise mechanism(s) by which T cells generate IL-17 are largely unknown.

In contrast to IL-17, the signaling mechanisms by which activated T cells drive expression of other cytokines such as IL-2 have been extensively analyzed (26–28). The TCR-CD3 complex

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1 The abbreviations used are: IL, interleukin; hIL, human IL; TCR, T cell receptor; NFAT, nuclear factor of activated T cells; CsA, cyclosporin A; Ab, antibody; PKC, protein kinase C; IP3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PLC, phospholipase; CHIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; PMA, phorbol 12-myristate 13-acetate; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase.

2 X. K. Liu, J. L. Clements, and S. L. Gaffen, unpublished data.
is activated following recognition of a peptide-major histocompatibility complex (MHC) complex on an antigen-presenting cell. TCR-CD3 signaling alone only causes relatively small amounts of IL-2 to be released. For optimal IL-2 production, further costimulation by CD28 interacting with B7-1 or B7-2 on the antigen-presenting cell is required. Together, these interactions lead to a highly complex signaling program, which causes nuclear import of several key transcription factors, including nuclear factor of activated T cells (NFAT), NF-κB, and AP-1, which ultimately activate transcription of the IL-2 gene. Specifically, engagement of the TCR-CD3 complex leads to activation of the tyrosine kinases ZAP-70, p56-Lck, and Itk. These in turn phosphorylate adapter molecules such as SLP76 and LAT, which bind to and recruit various key signaling enzymes. In particular, phospholipase Cγ1 (PLCγ1) becomes phosphorylated, leading to generation of the second messenger molecules inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ triggers rapid Ca²⁺ release from intracellular stores, which activates the phosphatase calcineurin, permitting dephosphorylation and nuclear import of the transcription factor NFAT (reviewed in Ref. 26). DAG activates protein kinase C pathways, particularly PKCθ, which leads to activation of the transcription factor NF-κB. However, signaling through CD28 is necessary for maximal activation of NF-κB. A recently discovered key mediator of CD28 signaling is the scaffold protein CARMA1, which assembles numerous signaling molecules in a complex necessary for NF-κB activation (29, 30). In addition, signaling through CD3 and CD28 activates MAPK pathways through Ras and JNK, leading to activation of the c-Fos and c-Jun, transcription factors that together comprise the AP-1 DNA binding complex. Together, NFAT, NF-κB, and AP-1 act in concert on the IL-2 proximal promoter, resulting in potent transcription of the IL-2 gene (reviewed in Refs. 27 and 31).

In the present study, we examined mechanisms of TCR-induced IL-17 production in a tissue culture model. First, we found that, in contrast to mouse cells, CD28 costimulation triggers a substantial increase in IL-17 production in human T cells. Moreover, both the calcineurin/NFAT pathway and MAPK pathways are required for IL-17 production, and we have identified two NFAT binding sites within the proximal promoter that are critical for TCR-mediated regulation of the human IL-17 gene.

**EXPERIMENTAL PROCEDURES**

**Cell Isolation, Tissue Culture, and ELISA**—Peripheral blood was purchased from Biological Specialty Corp. (Colmar, PA), and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Sigma) gradient centrifugation. 10⁶ cells were cultured in 96-well plates in the presence of anti-CD3 (OKT3), and/or anti-CD28 (Caltag Laboratories, Burlingame, CA) or PMA/ionomycin (Biomol International, Plymouth Meeting, PA) for 24 or 48 h. Jurkat and Hut102 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Gemini Bioproducts, Woodland, CA), 1% penicillin/streptomycin, and 1-glutamine (Invitrogen). Supernatants were analyzed by ELISA per the manufacturer’s instructions (BioSource Europe S.A., Nivelles, Belgium).

**PCR, Plasmids, Transfections, and Luciferase Assays**—The transcriptional start point of the IL-17 gene was determined by 5′-RACE (data not shown). PCR primers were designed to subclone sequences 1125 bp upstream of the transcriptional start site based on data in the human genomic DNA data base (NT_007592), and the resulting product was confirmed by sequencing. Oligonucleotide primers used in cloning (MWG Biotech, High Point, NC) were as follows: h17p(-1125)-Luc, (5′-AGCGGCTTGAGTCACCCC-3′); h17p(−159)-Luc, (5′-AGCGGCTTGAGTCACCCC-3′); h17p(TATA)-Luc construct was created by annealing two oligonucleotides, 5′-CGCCCAAAGCTTAAAATTTGTTTCATA-3′; and h17p(−924)-Luc, (5′-AGCGGCTTGAGTCACCCC-3′). The mutant NFAT sequence was 5′-GCCGCTTGAGTCACCCC-3′ (mutation site underlined). The anti-NFAT Abs (sc-1149), anti-NFATc1 (sc-7394), and anti-NFATc2 (sc-7296) and an isotype-matched nonspecific Ab used for supershifting were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Western blotting was performed as described previously (33). Abs to phosphorylated JNK (Ab 9251), phosphorylated p38 (Ab 9212), and p38 (Ab 9212) were purchased from Cell Signaling Inc. (Beverly, MA). Abs to phosphorylated ERK (sc-7383) and ERK (sc-154) were obtained from Santa Cruz Biotechnology.

CHIP assays were performed using a chromatin immunoprecipitation assay kit (Upstate Biotechnology, Inc., Lake Placid, NY), following the manufacturer’s protocol with modifications. In brief, 5 × 10⁶ Hut102 cells were stimulated with PMA/ionomycin for 30 min and then fixed with 1% formaldehyde (final concentration) for 15 min at room temperature. Cross-linking reactions were quenched with 250 μM glycine. Cells were briefly lysed with 0.4% Nonidet P-40, and nuclei were collected by centrifugation. 200 μL of SDS lysis buffer was added to the nuclei for 10 min on ice. Cell lysates were sonicated to shear the chromatin into 200–500-bp fragments, and debris was removed by centrifugation. For immunoprecipitation, 2 μg of anti-NFATc1 or anti-NFATc2

**FIG. 1. IL-17 is produced by peripheral blood mononuclear cells in response to CD3 and CD28 cross-linking.** Human PBMCs were cultured in the presence of anti-CD3 (2 μg/ml) and/or anti-CD28 (1 μg/ml) Abs or PMA/ionomycin (20 and 250 ng/ml, respectively) for 24 or 48 h. Supernatants were analyzed in triplicate for human IL-17 by ELISA. S.D. values are shown.
**RESULTS**

**Human IL-17 Is Produced by Primary Lymphocytes Stimulated through the TCR**—Since the signals that regulate IL-17 production remain poorly defined, we examined IL-17 secretion in primary human PBMCs in response to standard T cell stimuli, including agonistic Abs to CD3 and CD28. Upon receptor cross-linking for 24 or 48 h with anti-CD3 Abs alone, PBMCs secreted detectable levels of IL-17, as assessed by ELISA (Fig. 1). Stimulation with anti-CD28 Abs alone did not induce IL-17 production. However, signaling from both anti-CD3 and anti-CD28 markedly enhanced IL-17 production (2.5- or 4-fold at 24 and 48 h, respectively). PMA/ionomycin treatment, which bypasses TCR signaling by mimicking the second messengers IP₃ and DAG produced by PLCγ, also resulted in potent IL-17 production. This pattern of regulation was strikingly different from that observed in mouse primary cells, in which costimulatory signaling from CD28 further increases IL-17 production.

**The 1-kb Region Upstream of the IL-17 Gene Is Biologically Active in Human T Cell Lines**—The expression of most T cell-derived cytokines is regulated at least in part by the initiation of transcription. To explore regulation of IL-17 transcription, the transcriptional start point was first determined by 5'-RACE (data not shown and Fig. 2A). Interestingly, the transcriptional start point we identified was slightly different from those previously reported (16, 19) and was located 51 bases downstream of transcriptional start point using PCR primers based on sequence information from the human genomic data base. According to results from the AliBaba2.1 binding site prediction program, this sequence indicated that human IL-17 is induced following TCR stimulation and that costimulatory signaling from CD28 further increases IL-17 production.

**NFAT Regulates the IL-17 Gene Promoter**

A structure of the putative human IL-17 promoter. The transcriptional start point of the human IL-17 gene was determined by 5'-RACE analysis (position -37, boxed) and differs slightly from that originally identified (position -45). -1.2 kb of 5'-flanking sequence upstream of the transcriptional start point was cloned from genomic DNA. A number of predicted transcription factor binding sites were identified, including AP-1 (blue), NF-kB (green), and NFAT (red), as indicated. The TATA box and translation starting point (underlined) are also shown. The bars above and below the sequence from -232 to -71 indicate sequences of double-stranded oligonucleotide probes 1–11 used for DNA binding analysis (see also Fig. 6). B, the 5'-proximal region of the human IL-17 gene or its TATA box was subcloned into the pGL3-enhancer vector (termed h17p(-1125)-Luc and h17p(TATA)-Luc, respectively). Plasmids were transfected in triplicate into Jurkat cells and stimulated with anti-CD3 Abs only weakly enhances IL-17 secretion (Fig. 2B). Although the increase was only about 2-fold, this finding was highly reproducible and is probably attributable to a high background of luciferase activity commonly observed

IgG1 were incubated with cell lysates overnight at 4 °C with rotation. 30 μl of Protein G beads (Roche Applied Science) was added and incubated for 1 h at 4 °C with rotation. Beads were then washed with low salt immune complex wash buffer, high salt immune complex wash buffer, LiCl immune complex wash buffer, and TE for 3–5 min in sequence. DNA-protein complexes were eluted with 500 μl of elution buffer, and cross-links were reversed at 65 °C overnight in the presence of 20 μl of 5 μl of proteinase K for 1 h at 45 °C, and DNA was recovered by phenol/chloroform extraction and ethanol precipitation in the presence of 20 μg of glycogen (Roche Applied Science). Specific DNA was amplified by PCR with primers spanning -159 to +34 of the IL-17 promoter.

**FIG. 2. The IL-17 putative promoter is active in Jurkat cells following T cell receptor activation.** A, structure of the putative human IL-17 promoter. The transcriptional start point of the human IL-17 gene was determined by 5'-RACE analysis (position -37, boxed) and differs slightly from that originally identified (position -45). -1.2 kb of 5'-flanking sequence upstream of the transcriptional start point was cloned from genomic DNA. A number of predicted transcription factor binding sites were identified, including AP-1 (blue), NF-kB (green), and NFAT (red), as indicated. The TATA box and translation starting point (underlined) are also shown. The bars above and below the sequence from -232 to -71 indicate sequences of double-stranded oligonucleotide probes 1–11 used for DNA binding analysis (see also Fig. 6). B, the 5'-proximal region of the human IL-17 gene or its TATA box was subcloned into the pGL3-enhancer vector (termed h17p(-1125)-Luc and h17p(TATA)-Luc, respectively). Plasmids were transfected in triplicate into Jurkat cells and stimulated with anti-CD3 (2 μg/ml) and/or anti-CD28 (1 μg/ml) or with PMA (20 ng/ml) and ionomycin (250 ng/ml). Luciferase activity was normalized to the internal control plasmid Renilla luciferase, and S.D. values are shown.

**TABLE**

| Condition       | Luciferase Activity | Normalized Luciferase Activity |
|-----------------|---------------------|--------------------------------|
| Unstim.         | 0.5                 | 1                              |
| anti-CD3        | 1.0                 | 2                              |
| anti-CD28       | 1.5                 | 3                              |
| anti-CD28+anti-CD28   | 2.0              | 4                              |
| PMA+Iono.       | 2.5                 | 5                              |

This putative IL-17 promoter was subcloned upstream of a luciferase reporter gene (termed h17p(-1125)-Luc), and its activity was examined in the Jurkat human T cell line. In Jurkat cells transfected with h17p(-1125)-Luc, stimulation with anti-CD3 Abs caused an increase in reporter gene activity (Fig. 2B). Although the increase was only about 2-fold, this finding was highly reproducible and is probably attributable to a high background of luciferase activity commonly observed
using pGL3-based luciferase vectors (34). Similar to primary cells, the reporter gene activity induced by anti-CD3 was further enhanced by cross-linking with anti-CD28 Abs but not by anti-CD28 alone. This induction is specific to elements within the promoter, because a construct containing only the TATA box derived from the hIL-17 promoter did not show reporter gene activity upon CD3 or CD3/CD28 cross-linking and was only slightly enhanced by PMA/ionomycin stimulation. Thus, activity of the h17p(−1125)-Luc construct appears to mirror regulation of IL-17 in primary human T cells.

Calcineurin/NFAT and MAPK Signaling Pathways Are Involved in IL-17 Transcriptional Regulation—As an initial step to exploring potential signaling pathways involved in IL-17 regulation, we first assessed the effects of several pharmacological inhibitors known to block production of both IL-2 and IL-17. We transfected Jurkat cells with the IL-17 promoter (i.e. h17p(−1125)-Luc), stimulated with anti-CD3/anti-CD28 Abs with or without inhibitors, and then compared luciferase activities with mock-treated samples. Cyclosporin A (CsA) acts by blocking the phosphatase calcineurin, which is responsible for activating the NFAT family of transcription factors (reviewed in Ref. 35). At a dose of 100 nM CsA, IL-17 promoter activity was largely blocked (Fig. 3A), consistent with other work that implicates calcineurin in IL-17 gene regulation (23). Subsequently, we examined MAPK pathways using the inhibitors SP600125 (which blocks JNK and p38) and PD98059 (which blocks ERK) (Fig. 3B). Reporter gene activity was also blocked by both SP600125 and PD98059, indicating that the MAPK pathway is involved in IL-17 regulation. In all cases, these inhibitory effects were not due to nonspecific cellular toxicity, since all cells were equally viable throughout the experiment (data not shown). Furthermore, in EMSA experiments, the CsA inhibitor specifically blocked DNA binding of NFAT but not AP-1 (Fig. 3C). Conversely, the JNK/p38 and ERK inhibitors specifically blocked DNA binding of AP-1 but not NFAT. These data indicate that TCR-mediated activation of the calcineurin and MAPK pathways is upstream of IL-17 promoter activity.

A highly valuable feature of the Jurkat cell line is the availability of subclones specifically defective in particular TCR signaling intermediates. Therefore, to further define signaling pathways involved in IL-17 transcriptional regulation, the h17p(−1125)-Luc construct was transfected into Jurkat cells defective in p56-Lck (JCaM1.6 (36, 37)), PLCγ1 (JCaM1.6 (36, 37)), PLCγ1 (JPM50.6 (29)). The tyrosine kinase p56-Lck acts early in the TCR-CD3 signaling cascade and lies upstream of nearly all CD3-dependent signals. Thus, as expected, h17p(−1125)-Luc displayed no activity in Lck− cells following TCR stimulation (Fig. 4A) (whereas the internal Renilla luciferase control was expressed at normal levels; data not
PLCγ1 is a phospholipase responsible for the generation of the DAG and IP3 messengers, which are in turn required for PKC- and calcium-dependent signals of the TCR, including both NF-κB and NFAT. Consequently, in Jγ1 cells, all signals downstream of PLCγ are absent. No activity of the hIL-17 promoter was observed in PLCγ1-deficient Jurkat cells. CARMA1 is a recently identified scaffold molecule that mediates protein kinase C (PKC)-induced NF-κB activation and hence is crucial for CD3/CD28 costimulation. Notably, however, CARMA1 is not necessary for CD3-mediated activation of NFAT (29). Whereas the hIL-17 promoter was fully active in CARMA1-deficient cells in response to CD3 cross-linking, the contribution of CD28 cross-linking to promoter activity was eliminated (Fig. 4A). These data indicate that the contribution from CD3 is probably via NFAT, which is clearly important for
hIL-17 promoter activity, whereas the contribution of CD28 depends on CARMA1.

To obtain more direct evidence that the MAPK and NFAT pathways indeed act on the hIL-17 promoter, wild type Jurkat cells were co-transfected with the h17p(-1125)-Luc construct together with a 5-fold excess of Ras (to activate MAPK pathways) or NFATc1. Ras showed a marked stimulatory effect on the promoter, both at basal levels and after stimulation (Fig. 4C). Similarly, when NFATc1 was co-transfected with the hIL-17 promoter-luciferase construct, the promoter also showed enhanced activity. Thus, the Ras and NFAT pathways can transactivate the IL-17 promoter.

Two Functional NFAT Binding Sites Are Located within the Minimal IL-17 Regulatory Region—To further define the regulatory sites within the IL-17 promoter, we created a series of truncations from the 5’ end of the promoter (Fig. 5A). These constructs were assayed for function in Jurkat cells stimulated with anti-CD3/anti-CD28 Abs (B) or PMA/ionomycin (C). Luciferase activities were measured as described in Fig. 2. S.D. values are shown.

Fig. 5. The minimal IL-17 regulatory elements fall into a 232-bp region. A, the series of truncations from the 5’ end of the IL-17 promoter was created as diagrammed here. B and C, Jurkat cells were transfected in triplicate with the indicated IL-17 promoter deletions and R-luciferase and stimulated with anti-CD3/anti-CD28 Abs (B) or PMA/ionomycin (C). Luciferase activities were measured as described in Fig. 2. S.D. values are shown.

Fig. 6. Two functional NFAT binding sites are located within the minimal IL-17 regulatory region. A, Jurkat cells were stimulated with nothing (U) or anti-CD3 and/or anti-CD28 Abs. NS, nonspecific bands. Nuclear extracts were subjected to EMSA using double-stranded oligonucleotide probes as indicated in the legend to Fig. 2A (probes 2, 3, 7, and 8 are shown; lanes 1–16) or consensus AP-1 or NFAT probes (lanes 17–20). B, nuclear extracts from Jurkat cells treated with nothing (U; lane 1) or anti-CD3/anti-CD28 Abs (lanes 2–9) were subjected to EMSA with probe 3 (see Fig. 2A). Competition assays were performed using a 100-fold excess of unlabelled oligonucleotide (lane 3), consensus NFAT (lane 4), or mutant NFAT (lane 5) oligonucleotides. Abs specific for NFAT, NFATc1, NFATc2, or isotype-matched control Abs were incubated with nuclear extracts prior to EMSA (lanes 6–9). Similar results were obtained using probe 7, although the intensity of DNA binding was considerably weaker (data not shown).
(i.e. enhanced binding after stimulation with anti-CD3 or anti-CD3/anti-CD28 Abs but not anti-CD28 Abs alone) were probes 3 and 7 (Fig. 6A and data not shown), which both contained predicted NFAT sites (Fig. 2). It is noteworthy that specific inducible binding activities were not observed in the probes containing the predicted AP-1 sites (probes 4, 5, 8, and 9) (Fig. 6A and data not shown), suggesting that Ras/MAPK probably acts indirectly rather than triggering direct binding of AP-1 to the promoter (see “Discussion”).

To confirm that probes 3 and 7 contain genuine NFAT binding sites, we performed both competition and “supershift” assays. As shown in Fig. 6B, the DNA binding activity in probe 3 could be competed with a 100-fold excess of unlabeled probe 3 or a consensus NFAT probe but not by a mutant NFAT probe (lanes 3–5). Furthermore, Abs specific for NFAT, NFATc1, and NFATc2 either impaired DNA binding activity or supershifted the probe (lanes 6, 8, and 9), whereas a nonspecific antibody did not (lane 7). A similar pattern of DNA binding and competition was observed for probe 7, although binding was qualitatively weaker to this probe (Fig. 6A and data not shown). These results confirmed that the two predicted NFAT sites in the −232 to −159 region are indeed bona fide NFAT binding elements.

To determine whether these NFAT sites are important for regulating promoter activity, we mutated one or both sites and assessed the consequences to DNA binding and luciferase activity. First, to confirm these mutations interfered with NFAT binding, we tested them for the ability to compete with a consensus NFAT probe in EMSA (Fig. 7A). Binding to a consensus NFAT probe could be competed with a 100-fold excess of unlabeled NFAT (lane 3), probe 3 (lanes 4 and 5), or probe 7 (lanes 8 and 9). However, mutated versions of the NFAT consensus (lane 12), probe 3 (termed 3m) or probe 7 (7m) did not compete for the binding, even at a 200-fold excess (lanes 6 and 7 and lanes 10 and 11). Second, we introduced these NFAT mutations into the minimal −232 promoter construct (termed h17p(−232NFATm1)-Luc, h17p(−232NFATm2)-Luc, and h17p(−232NFATm1m2)-Luc) and transfectated them into Jurkat cells (Fig. 7B). Mutation of either NFAT site alone decreased luciferase activity only marginally. However, mutations at both sites reduced promoter activity to a level almost identical to that of the h17p(−159)-Luc construct. Therefore, TCR-induced activity in the −232 proximal promoter appears to be completely dependent on the two NFAT sites.

Although no human T cell lines have been found previously to produce IL-17, we discovered a derivative of the cell line Hut102 that secretes IL-17 at constitutively high levels (Fig. 8A). Consequently, we tested the IL-17 promoter deletion series in these cells, which exhibited a highly similar pattern as in Jurkat cells (Fig. 8B). Because treatment of Hut102 cells with PMA and ionomycin or a combination of PMA and anti-CD3/anti-CD28 Abs did not enhance IL-17 production further (Fig. 8A), it was not surprising that the IL-17 promoter activity was also not inducible by these treatments (Fig. 8B). Since the IL-17 promoter is constitutively active in Hut102 cells, we hypothesized that binding of NFAT to the IL-17 promoter would also be constitutive. Indeed, nuclear extracts from Hut102 cells showed constitutive binding to the NFAT probe (Fig. 6A, lanes 3 and 4), which could be competed with excess unlabeled NFAT oligonucleotide (lane 5). Finally, to show that NFAT occupies this IL-17 promoter region in vivo, we performed CHIP assays in Hut102 cells. Importantly, Abs to NFATc1 efficiently immunoprecipitated all NFATc1 isoforms, whereas a control Ab did not (Fig. 8D). Next, we performed CHIP with anti-NFATc1 Abs or control IgG Abs and then amplified the proximal promoter region by PCR (Fig. 8E). There was detectable background amplification in the nonspecific control immunoprecipitates, which is commonly observed in CHIP experiments. However, the signal was much stronger in samples immunoprecipitated with NFATc1-specific Abs (lane 2). Thus, the proximal region of the IL-17 promoter binds to NFATc1 in vivo.

**FIG. 7.** NFAT binding sites are important for DNA binding and promoter activity in the minimal IL-17 promoter. A, nuclear extracts from Jurkat cells stimulated with anti-CD3/anti-CD28 Abs for 30 min were incubated with a consensus NFAT probe and competed with a 100-fold excess of unlabeled NFAT oligonucleotide (lane 3), a 100- or 200-fold excess of probe 3 (lanes 4 and 5), probe 7 (lanes 8 and 9), or mutated versions of these (3m (lanes 6 and 7), 7m (lanes 10 and 11), and NFATm (lane 12)). B, the NFAT mutations were introduced singly or together into the −232 minimal promoter construct (termed h17p(−232NFATm1), h17p(−232) NFATm2, and h17p(−232) NFATm1m2), and luciferase assays were performed as in Fig. 2.

**DISCUSSION**

IL-17 is part of a relatively new cytokine family that exhibits potent inflammatory activities, both alone and in concert with other cytokines. Elevated expression of IL-17 has been reported in many clinical conditions, particularly rheumatoid arthritis and lung infections (39, 40). Compared with extensive studies of the biological activities of IL-17, considerably less is known about regulation of IL-17 itself. In this study, we have provided evidence that NFAT is a central regulator of T cell-mediated IL-17 gene transcription, and we identify two crucial NFAT elements located in the IL-17 proximal promoter.

A key distinguishing feature of NFAT is its regulation by Ca\(^{2+}\) and the Ca\(^{2+}\)/calmodulin-dependent serine phosphatase calcineurin. In resting cells, NFAT proteins are phosphorylated
and reside in the cytoplasm. Upon stimulation, they are dephosphorylated by calcineurin, whereupon they translocate to the nucleus and become transcriptionally active. When Ca\textsuperscript{2+} entry is prevented or calcineurin activity is inhibited, NFAT is rephosphorylated by NFAT kinases, such as CK1 and GSK3, and rapidly leaves the nucleus, thus terminating NFAT-dependent gene expression (41–43). Several lines of evidence indicate that NFAT is involved in regulation of IL-17 expression. First, CsA, an immunosuppressive drug that specifically blocks the enzymatic activity of calcineurin, has been shown to block IL-15-induced IL-17 production (23). Second, the cAMP/protein kinase A pathway has been implicated in IL-17 regulation, and this pathway is known to regulate NFAT nuclear localization through its action on glycogen synthase kinase-3 (17, 18, 44). Consistent with this, we found that cyclosporin A (CsA) completely blocks activity of the putative IL-17 promoter in Jurkat cells (Fig. 3), and exogenously expressed NFATc1 further enhances IL-17 promoter activity (Fig. 4C). We showed that both NFATc1 and NFATc2 could be supershifted in extracts from activated cells (Fig. 6B) and that NFATc1 occupies the promoter in vivo (Fig. 8E). Because the available anti-NFATc2 Abs do not work for immunoprecipitation, we could

**FIG. 8.** Hut102 cells secrete IL-17 constitutively, and the −222 to −159 region is also crucial for promoter activity in these cells. A, Hut102 cells were stimulated with nothing (U), PMA, and ionomycin or PMA and anti-CD3/anti-CD28 Abs for 24 h, and IL-17 was measured in culture supernatants by ELISA. B, Hut102 cells were transfected with the reporter constructs h17p(−1125)-Luc, h17p(−232)-Luc, h17p(−159)-Luc, or h17p(TATA)-Luc together with R-luciferase, and luciferase activities were assessed as described in Fig. 2. C, Jurkat (lanes 1 and 2) or Hut102 cells (lanes 3–5) were stimulated with nothing (U) or with anti-CD3/anti-CD28 Abs (3/28). In lane 5, nuclear extracts from Hut102 cells were competed with a 200-fold excess of unlabeled NFAT probe. Note that all panels in this figure were derived from the same film. D, Hut102 cells were lysed and immunoprecipitated (IP) with decreasing concentrations of Abs to NFATc1 (lanes 1–3), control IgG Abs (lanes 4 and 5), or no Abs (N; lane 6). Immunoprecipitates or whole cell lysates (WCL) were separated by SDS-PAGE and Western blotted (WB) with anti-NFATc1 Abs. Note that all panels in this figure were derived from the same film. E, Hut102 cells were stimulated with PMA/ionomycin and subjected to CHIP analysis using anti-NFATc1 or control IgG Abs for immunoprecipitation. The region between −159 and +34 of the IL-17 genomic locus was then amplified by PCR. This result is representative of multiple experiments.
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not perform similar CHIP experiments to confirm its occupancy of this promoter.

Costimulation of human primary T cells with anti-CD28 Abs further enhances levels of TCR-induced IL-17 expression (Fig. 1). This finding was unexpected, since costimulation by anti-CD28 Abs had only a very mild enhancing effect on CD3-induced IL-17 in mouse primary cells.2 It is possible that regulation of IL-17 may differ by species. Alternatively, the mouse studies were performed in cells taken from spleen and lymph nodes, whereas the human studies were done in PBMCs, and the latter may be more sensitive to CD28 costimulation for unknown reasons. The transcription factor NF-κB is crucial for CD28-mediated costimulation of the IL-2 gene, but CD28 costimulation-induced IL-17 production does not appear to involve NF-κB directly. Although the proximal promoter of the human IL-17 gene contains an NF-κB consensus site at position −643, no analogous site could be identified in the mouse promoter (data not shown). Moreover, deletion constructs of the human IL-17 promoter that lack this NF-κB site are still responsive to CD28 costimulation in Jurkat cells (Fig. 5). Although it is formally possible that there are cryptic NF-κB sites located within the minimal promoter region, none was revealed in our scanning oligonucleotide EMSA analysis (Fig. 6 and data not shown). The hIL-17 promoter is fully active in CARMA1(−/−) Jurkat cells in response to CD3 cross-linking alone (Fig. 4A), but CD28 costimulation does not increase promoter activity further. This loss of costimulation implies that CARMA1 is important for maximizing human IL-17 production. Another possible target downstream of CARMA1 in mediating CD28-induced IL-17 expression is JNK (45), which is consistent with the ability of the JNK inhibitor SP600125 to block IL-17 production (Fig. 3).

In this regard, MAPK pathways appear to be involved in IL-17 gene regulation, as indicated by both pharmacological inhibitors and cotransfection assays with Ras (Figs. 3 and 4). Not only did the ERK and JNK/p38 inhibitors block IL-17 promoter activity (Fig. 3), but overexpressed Ras enhanced it (Fig. 4C). It is still not clear what the major target of the MAPK pathway is with respect to IL-17. AP-1 is a key transcription factor controlled by MAPK pathways and often forms a strong cooperative complex with NFAT in cytokine regulation (reviewed in Refs. 46 and 47). Although we identified several putative AP-1 binding sites in the hIL-17 promoter, we could not demonstrate inducible AP-1 DNA binding activity by EMSA (Fig. 6A and data not shown). Alternatively, the MAPK pathway controls other transcription factor activities (48–50) that may play roles in IL-17 regulation. We recently found that Ets-1 overexpression does not substantially activate the IL-17 promoter in reporter assays,3 but we cannot rule out the possible involvement of the numerous other members of the Ets family that are expressed in lymphocytes. A more attractive possibility is that the MAPK pathway may affect NFAT activity. For example, it has been shown that NFATc2 and AP-1 regulate expression of NFATc1 (50, 51), and thus MAPK signaling can amplify NFAT-dependent functions. Consequently, cross-talk between the NFAT and MAPK signaling pathways may act upstream of the IL-17 promoter itself. Clearly, however, the MAPK pathway is important for TCR-mediated IL-17 gene regulation, and future efforts will be directed to determining the mechanism by which this occurs.

A caveat of Jurkat cells as a model system is that they do not secrete IL-17 under any conditions of stimulation we have tried, including CD3, CD28, IL-23, or PMA/ionomycin. It may be that additional chromatin modifications are required for IL-17 production. Alternatively, this could be due to one of the molecular defects inherent to these cells (e.g., the phosphatases PTEN and SHIP are defective, leading to constitutive phosphatidylinositol 3′-kinase activation (52)). Nevertheless, the IL-17 promoter is active in Jurkat cells in a manner consistent with its production by normal T cells and thus provides a model system in which to examine CD3-mediated signaling requirements that lead to activation of the IL-17 gene. Another valuable feature of the Jurkat cell line is the availability of sublines specifically defective in particular TCR signaling intermediates, which enables investigation of the genetic basis for TCR signaling pathways involved in IL-17 regulation (Fig. 4).

We recently discovered that the Hut102 T cell line secretes high levels of IL-17 constitutively (Fig. 8A), thus providing a means to validate results obtained in Jurkat cells. Importantly, all of our findings in Hut102 were highly consistent with the results in Jurkat cells. Specifically, the region of the IL-17 promoter critical for activity lies between −232 and −159 (Fig. 8B), and nuclear extracts from Hut102 cells bind to NFAT sites constitutively (Fig. 8C). This cell line will be very useful in future studies to probe IL-17 gene regulation and in particular to address the question of why Jurkat cells fail to express IL-17.

In summary, this work defines proximal cis-acting promoter elements in the IL-17 promoter that regulate its responsiveness to TCR signaling, and we specifically identified two critical NFAT sites in the −232 to −159 region. Increasingly, it is clear that IL-17 plays a pathogenic role in inflammatory diseases, particularly arthritis and lung diseases (reviewed in Refs. 5 and 40). Understanding the molecular mechanisms by which production of IL-17 is controlled may offer new opportunities for therapeutic intervention for diseases involving this cytokine.

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