Identification of Common Transcriptional Regulatory Elements in Interleukin-17 Target Genes*\textsuperscript{5}

Fang Shen\textsuperscript{5}, Zihua Hu\textsuperscript{5}, Jaya Goswami\textsuperscript{5}, and Sarah L. Gaffen\textsuperscript{5,1*}

From the \textsuperscript{5}Department of Oral Biology, School of Dental Medicine, \textsuperscript{5}Department of Microbiology and Immunology, School of Medicine and Biomedical Sciences, and \textsuperscript{1}Center for Computational Research, Department of Biostatistics, School of Medicine and Biomedical Sciences, University at Buffalo, State University of New York, Buffalo, New York 14214

Interleukin (IL)-17 is the founding member of a novel family of inflammatory cytokines. Although produced by T cells, IL-17 activates genes and signals typical of innate immune mediators such as tumor necrosis factor (TNF)-\(\alpha\) and IL-1\(\beta\). Most IL-17 target genes characterized to date are cytokines or neutrophil-attractive chemokines. Our recent microarray studies identified an acute phase response gene, 24p3/lipocalin 2, as a novel IL-17-induced gene. Here we describe a detailed analysis of the 24p3 promoter. We find that, unlike cytokine or chemokine gene target genes, 24p3 is regulated primarily at the level of transcription rather than mRNA stability and that synergy between IL-17 and TNF activates genes and signals typical of innate immune mediators. Although produced by T cells, IL-17 communicates with the innate immune system to promote inflammation, which bridges the adaptive and innate immune systems. To date, six IL-17 family ligands (IL-17A-F) and five receptors (IL-17RA-IL-17RD and SEF) have been identified (1, 2). By far the best characterized is IL-17 (CTLA-8 or IL-17A), which is secreted primarily by CD4\(^+\) T helper cells. Recent reports have demonstrated that the IL-12 family cytokine IL-23, in conjunction with transforming growth factor \(\beta\) and IL-6, drives IL-17 expression in a unique CD4\(^+\) T cell subset, now termed “Th17” (3–5). Although produced by the adaptive arm of the immune system, IL-17 functions as a classic effector of innate immunity, similar to IL-1\(\beta\), TNF\(\alpha\), or Toll-like receptor agonists such as lipopolysaccharide (1). Specifically, IL-17 induces expression of many innate inflammatory mediators, including IL-6, CXC chemokines, acute phase proteins, granulocyte-colony stimulating factor, prostaglandin E2, and cyclooxygenase-2. IL-17 also synergizes potently with inflammatory cytokines such as TNF\(\alpha\), amplifying its effects by orders of magnitude (6, 7).

Thus, IL-17 is a means by which the adaptive immune system communicates with the innate immune system to promote inflammation.

Like most inflammatory cytokines, IL-17 plays opposing roles in vivo, depending on disease context (8). On one hand, considerable evidence links IL-17 to pathology in autoimmune disease, including rheumatoid arthritis, psoriasis, systemic lupus erythematosus, and Crohn disease (9, 10). Blocking IL-17 with antibodies or soluble receptors reduces inflammatory symptoms in animal models of arthritis such as collagen-induced arthritis, whereas excess IL-17 exacerbates disease (11–13). IL-17-deficient mice are also resistant to collagen-induced arthritis, and spontaneous arthritis in IL-1ra-deficient mice is dependent on IL-17 (14). In contrast, IL-17 is essential for mounting effective immune responses to many infectious organisms. In most cases, this has been linked to a failure to expand and/or recruit neutrophils (1, 15). Consequently, IL-17RA-deficient mice show an increased susceptibility to bacterial, fungal, and parasitic pathogens (16–18).

The process of inflammation is coordinated by soluble effectors, primarily cytokines and chemokines, which trigger vascular changes to activate and recruit leukocytes and other host defense mechanisms. In the early stages of inflammation, the innate immune system and a network of pro-inflammatory cytokines and chemokines promote phagocytosis and neutrophil recruitment to infected sites. Within days, innate immune mechanisms in turn activate the adaptive immune system to mount a specific memory response involving antigen-specific lymphocytes.

Interleukin (IL)\textsuperscript{2}-17 is a relatively newly described cytokine that bridges the adaptive and innate immune systems. To date, six IL-17 family ligands (IL-17A-F) and five receptors (IL-17RA-IL-17RD and SEF) have been identified (1, 2). By far the best characterized is IL-17 (CTLA-8 or IL-17A), which is secreted primarily by CD4\(^+\) T helper cells. Recent reports have demonstrated that the IL-12 family cytokine IL-23, in conjunction with transforming growth factor \(\beta\) and IL-6, drives IL-17 expression in a unique CD4\(^+\) T cell subset, now termed “Th17” (3–5). Although produced by the adaptive arm of the immune system, IL-17 functions as a classic effector of innate immunity, similar to IL-1\(\beta\), TNF\(\alpha\), or Toll-like receptor agonists such as lipopolysaccharide (1). Specifically, IL-17 induces expression of many innate inflammatory mediators, including IL-6, CXC chemokines, acute phase proteins, granulocyte-colony stimulating factor, prostaglandin E2, and cyclooxygenase-2. IL-17 also synergizes potently with inflammatory cytokines such as TNF\(\alpha\), amplifying its effects by orders of magnitude (6, 7).

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1 and 2.

Supported by the National Institutes of Health Grant AR050458 and the Arthritis Foundation. To whom correspondence should be addressed: 36 Foster Hall, 3435 Main St., Buffalo, NY 14214. Tel.: 716-829-2786; Fax: 716-829-3942; E-mail: sgaffen@buffalo.edu.

\(^{2}\) The abbreviations used are: IL, interleukin; LIX, lipopolysaccharide-inducible CXC chemokine; TFBS, transcription factor (TF) binding site; C/EBP, CAAT/enhancer-binding protein; EMSA, electrophoretic mobility shift assay; TNF, tumor necrosis factor; TSS, transcriptional start site; STAT, signal transducers and activators of transcription; MEF, murine embryonic fibroblast; FBS, fetal bovine serum; kb, kilobase(s); WT, wild type; Ab, antibody.
In the course of recent microarray studies, we found that another gene induced potently by IL-17 is 24p3, also known as lipocalin 2 or neutrophil gelatinase-associated lipocalin (19). Lipocalins are small, secreted proteins with a common tertiary structure that binds lipophilic molecules such as prostaglandins and cholesterol. 24p3 was originally identified as an acute phase response gene induced in the liver, mainly by inflammatory stimuli such as TNFα and lipopolysaccharide (20, 21). Diverse activities have been ascribed to 24p3, including roles in apoptosis, tissue involution, and ischemia-reperfusion injury (22–24). However, the most convincing function of 24p3 is its role in innate immune defense via iron sequestration. Most pathogenic organisms require host-derived iron for survival and have developed complex systems for its acquisition (for review, see Ref. 25). Consequently, an important mammalian host defense mechanism involves preventing bacterial access to free iron. 24p3 was recently shown to bind to catecholate-type siderophores and thereby block iron acquisition by certain types of bacteria (26). Consistent with this, 24p3-deficient mice are highly sensitive to organisms that use catecholate-type siderophores such as Escherichia coli (20, 27). These findings indicate that another antimicrobial signal regulated by IL-17 involves the acute phase response and control of bacterial access to free iron.

Despite its functional similarity to the IL-1 and TNF families, IL-17 and its receptor IL-17RA are unique in structure and sequence (28), and therefore, few predictions about molecular signaling pathways is typical of pro-inflammatory effectors such as TNFα, lipopolysaccharide, and IL-1β. Numerous experiments both in vitro and in vivo have shown that IL-17 exerts potent effects on neutrophils, primarily by regulating genes encoding chemokines, ICAM-1 (intercellular adhesion molecule 1), and granulopoietic cytokines such as granulocyte- and granulocyte-macrophage-colony stimulating factor (15). Knowing gene targets of signaling pathways allows inferences to be made about signaling pathways activated by the inducing receptor. Although few IL-17-induced genes have been characterized in detail, most of those examined contain an essential NF-κB DNA binding site in their proximal promoters (6, 29). Consistent with this, IL-17 has been shown to activate NF-κB nuclear import and DNA binding in certain cell types via the adaptor TRAF6 (30, 31). We recently showed that IL-17-induced activation of the IL-6 promoter involves NF-κB activation in conjunction with the CCAAT/enhancer-binding protein (C/EBP, also known as NF-IL6) transcription factor family (6). However, information about IL-17-mediated signaling is still very limited.

The present study describes a detailed analysis of the 24p3 proximal promoter and its activation by IL-17 and TNFα, in which we found that IL-17 uses both NF-κB and C/EBP to activate the 24p3 promoter. Given the similarity between the IL-6 and 24p3 promoters in terms of IL-17 regulation, we performed a computational analysis of transcription factor binding sites (TFBS) using multiple mouse and orthologous human IL-17 target genes. Results revealed statistical over-representation of TFBSs common to IL-17 target gene promoters, including NF-κB and C/EBP. Moreover, a phylogenetic comparison of IL-17-target gene promoters in human and mouse identified three subcategories of promoter based on TFBS location and usage. Strikingly, these promoter subgroups correlated with gene function, in that one category of promoter contained only chemokines, whereas another contained only immuno-regulatory molecules such as 24p3. These studies suggest that IL-17 may use different signaling cascades to specifically direct different categories of gene expression.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Reagents, Transfections, and Stimulations**—ST2 and MG63-E1 and murine embryonic fibroblasts (MEF) were cultured in α-minimum essential medium (Sigma), supplemented with 10% fetal bovine serum (FBS; Gemini Bioproducts, Woodland CA), penicillin, streptomycin, and L-glutamine (Invitrogen). Cells were transfected with the FuGENE 6 Transfection Reagent (Roche Applied Science). Recombinant human IL-17 and TNFα were from R&D Systems (Minneapolis, MN). For stimulation, ST2 or IL-17RKO MF cells were seeded on 10-cm plates at 70% confluence. After attachment (~16 h), cells were stimulated with the indicated cytokines for the designated time periods.

**Real-time PCR**—Real-time PCR was performed as described (19) with an iCycler IQ (Bio-Rad). Primer pairs were as follows: 24p3, 5’-CAG CTT TCA GAT GTA CAG CAC C-3’ and 5’-CAT GGC GAA CTT GTT GTA GTC-3’; CXCL chemokine ligand 1 (Groα/CXCL1), 5’-CAC CCA AAC CGA AGT CAT AG-3’ and 5’-AAG CCA CCA GGC TCC ACC AGA-3’; LIX/CXCL5, 5’-GTT CCA CAG TGC CCT AGC-3’ and 5’-GCG AGT GCA TTC CGC TTA-3’; C/EBPβ, 5’-TGC CAT GTA CGA CGA G-3’ and 5’-GCC GCT TTG TGG TTG CTG-3’; C/EBPδ, 5’-CCG ACC AGC ACT TCC TCT-3’ and 5’-CGA GGC TCA CGT AAC CGT-3’. For measuring RNA stability, ST2 cells were stimulated for 4 h without cytokines or with IL-17 (200 ng/ml). Cells were washed twice with phosphate-buffered saline and treated with actinomycin D (5 μg/ml, Sigma) for the durations indicated.

**Luciferase Assays**—Construction of the luciferase reporter assay plasmid containing 1.4 kb upstream of the 24p3 gene (24p3-1400-luc) based on sequence information from the genomic data base (accession number NC_000068) was described previously (19). Serial 5’ truncations of the 24p3 promoter were generated by PCR and subcloned in the MluI and XhoI sites of the pGL3 Basic reporter plasmid (Promega, Madison, WI). Upstream primers were: 24p3-1088-luc, 5’-CAG CTT TCA GAT GTA CAG CAC C-3’ and 5’-CAT GGC GAA CTT GTT GTA GTC-3’; 24p3-716-luc, 5’-GTT CCA CAG TGC CCT AGC-3’ and 5’-GCC GCT TTG TGG TTG CTG-3’; 24p3-144-luc, 5’-GCC GCT TTG TGG TTG CTG-3’. Site-directed mutations were performed using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) per the manufacturer’s instructions. Primers used to create the NF-κB mutation were:
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5'-AGA ATC CAA AGC CCT GCG AAT GTC CCT CTG GTC CC-3' and 5'-GAG GGG GAC CAG AGG GAC ATT CGC AGG GCT TTG GAT-3'. Primers used to create the C/EBP mutation were 5'-CAG CCC TTC CTG GCA CTT GGC CTT GCA CAG TTC CGA C-3' and 5'-AAC TGT GCA AGG CCA AGT GCC AGG AAG GGC TGC AGG G-3'(mutation sites are underlined).

For luciferase assays cells were seeded on 12-well plates (1 x 10^5 cells/well) and co-transfected with 0.2 µg of the 24p3 luciferase plasmid and 10 ng of Renilla luciferase plasmid as an internal standard. Cells were stimulated with cytokines for 6 h, and lysed, and supernatants were analyzed for luciferase using a Veritas Microplate luminometer (Turner Biosystems, Sunnyvale, CA).

EMSA—NF-κB EMSAs were performed as described (6) with 10 µg/lane nuclear extracts and 10^6 cpm/lane 32P-labeled double-stranded probe. The WT NF-κB probe (top strand) is 5'-AAG CCC TGG GAA TGT CCC TCT G-3', and the mutant NF-κB sequence is 5'-AAG CCC TGC GAA TGT CCC TCT G-3' (the mutation is underlined). The sequence of the WT C/EBP probe (top strand) is 5'-CTT CCT GCT GCT A-3', and the mutant C/EBP sequence is 5'-CTT CCT GGC ACT TGG CCT TGC A-3' (the mutation is underlined). The composite NF-κB/C/EBP composite long probe is 5'-AAG CCC TGG GAA TGT CCC TCT G-3', and 5'-AAG CCC TGC GAA TGT CCC TCT G-3'. Primers used to create the C/EBP mutation were 5'-CAG CCC TTC CTG GCA CTT GGC CTT GCA CAG TTC CGA C-3' and 5'-AAC TGT GCA AGG CCA AGT GCC AGG AAG GGC TGC AGG G-3' (mutation sites are underlined).

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shown, the 24p3 message appears to be very stable, and IL-17 stimulation does not alter the degradation kinetics of 24p3 mRNA. In contrast, IL-17 signals do mediate stability of the LIX/CXCL5 message, consistent with our previous findings (Fig. 1C) (19). Thus, IL-17 does not appear to control 24p3 mRNA degradation.

To determine whether production of the 24p3 protein correlates with mRNA production, we tested 24p3 protein expres-
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As expected, 24p3 protein expression showed a similar profile to mRNA expression. Specifically, the 24p3 protein was induced after stimulation with IL-17 alone and IL-17 with TNFα (Fig. 1D). Because 24p3 is a secreted protein, we also tested its expression in cell supernatants. Although IL-17 treatment by itself did not trigger high levels of 24p3 secretion, IL-17 + TNFα stimulation did so. Thus, ST2 cells produce and secrete 24p3 in response to inflammatory cytokines.

IL-17 Regulates the 24p3 Promoter via NF-κB and C/EBP—

These results indicated that IL-17 regulates 24p3 at the level of transcription initiation. To define regulatory elements in the 24p3 proximal promoter responsive to IL-17, we cloned 1.4 kb of genomic DNA upstream of the 24p3 transcriptional promoter (19). This putative promoter shows a 5–10-fold IL-17-inducible activity in all cell types tested (Fig. 2A). In contrast, a low dose of TNFα (2 ng/ml) stimulates 24p3 promoter activity only weakly (2-fold) (Fig. 2A). However, the 24p3 promoter shows synergistic activation (25–30-fold) in response to IL-17 combined with TNFα and is one of the only genes so far identified where synergistic signals between IL-17 and TNFα occur at the level of transcription initiation.
the promoter (6). The degree of inducibility of the 24p3 promoter was comparable in MC3T3-E1, MEF, and ST2 cells. Interestingly, the magnitude of IL-17-mediated induction of the 24p3 promoter is not as dramatic as what is observed at the mRNA level, which may be due to differences in assay sensitivity but may alternatively reflect contribution from other epigenetic control mechanisms such as chromatin modification. Nonetheless, transcriptional initiation is clearly a major means of activating 24p3 expression. We then performed a detailed analysis of the 24p3 proximal promoter. First, we compared conserved noncoding sequences within the mouse and human 24p3 loci to identify candidate DNA binding sites using the rVista program (Fig. 2B) (41). Several conserved sites were found, including a STAT site at position −642, an NF-κB site at −260, and a C/EBP site at −213 (Fig. 2B). To determine the minimal IL-17-responsive region within the promoter, we created a series of promoter deletions and tested their activities in ST2 cells (Fig. 2C). These studies revealed that the minimal IL-17-responsive promoter is contained within a −282-bp element upstream of the predicted transcriptional start site. The region between −282 and −716 also appears to contribute to optimal transcriptional capacity, perhaps due to the STAT site, although we have not yet pursued this further. However, the deletion at −213, which deletes the predicted NF-κB element, completely eliminated IL-17- and IL-17 + TNF-α-induced reporter activity. The essential role of this NF-κB site was verified by a point mutation within the −282 minimal promoter (Fig. 2D). Interestingly, mutation of the conserved C/EBP site also eliminated promoter activity, even in the presence of an intact NF-κB site (Fig. 2D). These data indicate that IL-17 activates the 24p3 promoter via both NF-κB and C/EBP and also that neither transcription factor (TF) drives detectable expression on its own. These data are consistent with our previous finding that overexpression of either the NF-κB p65 subunit or C/EBPβ or C/EBPδ can transactivate the 24p3 promoter (19) and that IL-17 does not activate linked NF-κB or C/EBP promoter elements alone (data not shown).

Transcription Factor Binding to the 24p3 Promoter—We next examined the binding of NF-κB to its target site in the 24p3 promoter by EMSA (Fig. 3, A–C). IL-17 stimulation of ST2 cells caused a rapid up-regulation of NF-κB DNA binding activity as early as 30 min. NF-κB binding was sustained for up to 8 h but disappeared by 24 h after IL-17 treatment (Fig. 3A). This DNA binding activity was confirmed to be NF-κB by cold oligonucleotide competition (lanes 7–8) and antibody supershifting (lanes 9–10). As expected, TNFα also stimulated NF-κB binding to DNA but with a somewhat different time course, in which DNA binding declined but was not completely absent at 24 h (Fig. 3B). When cells were treated with both cytokines, the overall magnitude of NF-κB binding was similar (Fig. 3C and data not shown) and was sustained at high levels for at least 24 h. These data suggest that, although IL-17 and TNFα do not synergize at the level of NF-κB at shortest time frames (consistent with prior findings (6)), longer-term signals at 8–24 h may involve a cooperative maintenance of NF-κB DNA binding.

We also examined C/EBP expression and DNA binding to the 24p3 C/EBP site after IL-17 stimulation. Because it is technically challenging to observe C/EBP binding by standard gel shift methods, we developed conditions such that we could observe C/EBP in EMSA (Fig. 4A) (42). As shown, there is considerable basal C/EBP DNA binding in untreated cells. However, increases in C/EBP binding were not observed until 2–4 h post stimulation and then declined between 8 and 24 h. Stimulation with TNFα alone induced C/EBP binding slightly at 4–8 h but not as potently as IL-17 (Fig. 4B). Treatment with both IL-17 and TNFα together induced a similar profile of C/EBP binding as IL-17 alone, indicating that cooperation with TNFα probably does not occur at the level of C/EBP DNA binding (Fig. 4C). Supershifting with isof orm-specific C/EBP antibodies suggested that both C/EBPβ and C/EBPδ formed part of the binding complex (lanes 9–11), which is in agreement with our prior findings that both C/EBPβ and C/EBPδ contribute to IL-17-mediated IL-6 expression (6).

The resolution in the C/EBP EMSA assays was not sufficient to determine conclusively which C/EBP isof orm(s) is contained within each complex or whether these are subtly altered upon IL-17 signaling. Therefore, we performed Western blotting on the same nuclear extracts used for EMSA with Abs to C/EBPβ and C/EBPδ (Fig. 4D). As shown, the faster-migrating isof orm of C/EBPβ is expressed constitutively in ST2 cells and is induced modestly by IL-17 stimulation. It is likely that this constitutes the basal levels of C/EBP binding observed in EMSAs. Another, slower-migrating C/EBPβ isof orm is induced strongly...
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![FIGURE 4. C/EBP binding to the 24p3 promoter is induced primarily by IL-17. ST2 cells were stimulated for the indicated times (1–24 h) with 200 ng/ml IL-17 (panel A, lane 1). Nuclear extracts were prepared and subjected to EMSA with a 32P-labeled oligonucleotide probe corresponding to the 24p3 C/EBP site. In lanes 1–2, NF-κB binds independently to both C/EBP sites in the 24p3 promoter. ST2 cells were unstimulated (lane 5) or stimulated with TNFα (lane 6), C/EBPβ (lane 7), or both cytokines (lane 8). For supershifting (lanes 9–11), nuclear extracts were incubated with Abs against C/EBPβ, C/EBPδ, or an isotype control. Bands corresponding to C/EBPβ or C/EBPδ were supershifted with Abs to p65, C/EBPβ, or C/EBPδ. Lane 12 shows the composite probe used in EMSA.](https://www.jbc.org/content/281/34/24144)

by IL-17 ~2 h post-stimulation, the same time point at which we observed a marked increase in C/EBP DNA binding in response to IL-17 (Fig. 4A, lane 3). C/EBPδ is present only at low levels in untreated ST2 cells but is induced potently 2 h after IL-17 stimulation and is sustained for up to 24 h. These results indicate that IL-17 signaling induces expression of both C/EBPβ and C/EBPδ in ST2 stromal cells, which correlates with their DNA binding activities at the 24p3 promoter. This pattern of expression is consistent with our previous results in MC3T3-E1 and MEF cells, in which IL-17 triggers expression of both C/EBPβ and C/EBPδ (6).

The NF-κB and C/EBP sites are spaced only 26 bp apart in the mouse 24p3 promoter. In other promoters regulated by inflammatory stimuli such as TNFα, C/EBP and NF-κB sites are also closely spaced and exhibit cooperative DNA binding (42–44).

To determine whether this was the case for 24p3, we synthesized a 67-bp oligonucleotide encompassing both sites as they exist in the 24p3 promoter and tested DNA binding in EMSA. Binding of NF-κB to the composite probe was not reduced by the presence of excess cold C/EBP probe and vice versa (Fig. 4E). Thus, despite their close proximity, it does not appear that either C/EBP or NF-κB requires the other TF for efficient DNA binding. This is further supported by the fact that these sites are located further apart in the mouse compared with the human 24p3 promoter, indicating that the relative distance between these sites is not conserved evolutionarily. Thus, NF-κB and C/EBP bind independently to their respective sites in the 24p3 promoter, but both are required for IL-17-induced promoter activity.

Common TF Binding Site Patterns in IL-17 Target Promoters—Results from these studies on 24p3 are strikingly reminiscent of our previous work on the IL-6 promoter, which also requires both NF-κB and C/EBP for IL-17-mediated transcription (6). This similarity prompted us to ask whether other IL-17 target genes show similar TFBS patterns in their proximal promoters. Accordingly, we performed a comparative analysis of 18 well-documented IL-17 target gene promoters to determine whether particular TFBSs are enriched in human and mouse species. Putative proximal promoter sequences from the mouse and human homologues of each gene were obtained from the Cold Spring Harbor Laboratory Mammalian Promoter data base (32, 33), defined as non-coding genomic DNA extracts were co-incubated with 100-fold excess of an unlabeled WT or mutant C/EBP (mC/EBP) site (mutation shown in Fig. 2D). For supershifting (lanes 9–11), nuclear extracts were incubated with Abs against C/EBPβ, C/EBPδ, or an isotype control. Bands corresponding to C/EBPβ or C/EBPδ were supershifted with Abs to p65, C/EBPβ, or C/EBPδ, or a control plasmid was used as a loading control. Lysates from HEK293 cells transfected with C/EBPβ, C/EBPδ, or a control plasmid were used as positive controls (lanes 6–8). For supershifting (lanes 9–11), nuclear extracts were incubated with a 32P-labeled oligonucleotide probe containing both the NF-κB and C/EBP sites in EMSA conditions developed for visualizing C/EBP (see “Experimental Procedures”). Extracts were competed with a cold WT or mutant NF-κB probe (lanes 6–7), cold WT, or mutant C/EBP probe (lanes 8–9) or supershifted with Abs to p65, C/EBPβ, C/EBPδ, or control IgG (lanes 11–14). Arrows indicate migration of both NF-κB and C/EBP DNA binding complexes. FP, free probe.
sequences 1000 bp upstream of the TSS. These promoters were scanned for 565 vertebrate position weight matrices from the professional TRANSFAC9.1 database using the CLOVER algorithm (34) (see “Experimental Procedures” for details). The frequency with which each TFBS was present in these promoters was compared statistically to randomized promoter sequences from the same species, which identified dozens of enriched TFBSs for both human and mouse genes (supplemental Tables 1 and 2). Next, to determine which of these many TFBSs were likely to be the most biologically relevant, we tabulated only those sites that were statistically enriched in both mouse and human promoters (Table 1). Not surprisingly, NF-κB sites were the most highly enriched in both mouse and human promoters (p < 0.001). C/EBP sites were also statistically overrepresented in human and mouse promoters. In addition to NF-κB and C/EBP, this analysis revealed that AP-1, Ikaros, and Oct-1 sites were also enriched in IL-17-inducible gene promoters. AP-1 and Oct-1 are commonly found to regulate immune genes, and IL-17 activates the mitogen-activated protein kinase pathway leading to AP1 activation. Ikaros is a well-known regulator of early lymphocyte development, and it was unexpected to find sites for this TF in IL-17 target genes. However, upon closer examination of the Ikaros binding site, we found that it closely resembled the NFKAPPA01 position weight matrix. Therefore, we presume the over-representation of Ikaros in this study is likely an artifact due to similarities in DNA recognition sites, and we did not analyze this TFBS further.

To predict whether individual TFs interact to co-regulate genes, we sought to determine whether combinations of TFBSs were present in the promoter sequences of the selected genes more often than in a randomly selected group of promoters from the same species (Table 2). We first calculated the background probability of combinations of two TFBSs in promoter sequences within 1 kb upstream of the transcriptional start site from 6600 mouse or 7350 human unique mRNA RefSeq genes. We then computed the number of genes whose promoter sequences contain combinations of two TFBSs among the selected genes. Finally, we calculated the probability of observing an equal or larger number of gene promoters in the selected genes with two TFBSs than in randomly selected sequences by chance by binomial distribution. Results indicated that the C/EBP and AP-1 sites were synergistically enriched with NF-κB sites. However, no enrichment was obtained for Oct-1 sites with any other TFBSs, and C/EBP and AP1 sites were synergistically enriched in mouse but not human promoters (Table 2). These results indicated that NF-κB is apparently found together with C/EBP and AP1 in IL-17 target genes, suggesting that IL-17-mediated signaling pathways activate TFs in a combinatorial manner to specifically up-regulate particular genes.

To determine phylogenetically conserved TFBSs among IL-17 target genes, the locations of TFBSs were compared for all 18 mouse and human promoters (Fig. 5). Interestingly, three categories of promoters emerged from this analysis based on the location and combination of TF sites within a given proximal promoter (Table 3, Fig. 5). Group 1 consisted of promoters containing proximal NF-κB and C/EBP sites. In general, the C/EBP sites were found within 300 bp of the TSS and the NF-κB sites within 600 bp of the TSS. Note that although the conservation of the NF-κB sites in the IkB gene was not identified in our analysis (data not shown), empirical studies have demon-
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TABLE 3
Functional categories of IL-17-induced target genes based on comparative analysis of proximal promoter sequences
Putative promoter sequences of 18 IL-17 target genes from mouse and human databases and locations of TFBSs were determined by mouse/human promoter alignments. Three apparent subgroups were identified based on NF-κB and C/EBP usage.

| Group | Functional category | Gene name | NF-κB | C/EBP |
|-------|---------------------|-----------|-------|-------|
| 1     | Immuno-regulatory molecules | 24p3/Lcn2, IL-6, MMP3, β-Defensin 2, G-CSF, COX2, lαβ7α" | At least 1 site within 600 bp | 1–2 sites within 300 bp of start |
| 2     | Chemokine | CXCL1, CXCL2, CXCL5, CCL2, CCL20 | 1–2 sites within 300 bp of start | Presence variable |
| 3     | Other | CXCL1, C/EBPβ, C/EBPα, Fas, MMP13, CCL7 | Not present | Presence variable |

*This alignment study did not identify NF-κB site in the lαβ7α promoter, but sites have been empirically identified.*

Strikingly, all the genes in Group 1 are immuno-regulatory molecules, such as cytokines and host defense factors including IL-6 and 24p3. Group 2 consisted of genes whose promoters contain proximal NF-κB sites within 300 bp of the TSS. These promoters did not commonly have C/EBP, AP1, or Oct1 sites. Strikingly, all the genes in this category were chemokines, suggesting a common regulatory motif for this type of molecule. The only chemokine not in this group is CCL7. Finally, Group 3 promoters appear to be NF-κB-independent and show little or no conservation in TFBS usage or location. Interestingly, C/EBPβ and C/EBPα are found in this category, which have both been implicated in IL-17-target gene regulation (see “Discussion”). Thus, the transcriptional regulatory machinery found in IL-17 target genes differs depending on the function of each gene within the immune system and may indicate alternate modes by which IL-17 signaling regulates different phases of immune responses.

**DISCUSSION**

IL-17 is a novel pro-inflammatory cytokine that contributes in numerous ways to host defense. Although its effects on chemokines and neutrophil activation are well documented, less is known about how IL-17 controls other aspects of innate immunity such as the acute phase response. Here we describe mechanisms of IL-17-induced gene regulation of the acute phase response gene, 24p3, which controls infection by limiting microbial access to free iron (25). We find that IL-17 regulates 24p3 primarily through initiation of transcription, with no apparent contribution to mRNA stability. The minimal IL-17-responsive region within the 24p3 proximal promoter lies 282 bp from the transcriptional start site, and IL-17-induced transcription requires a conserved NF-κB site as well as a C/EBP binding site. Although both sites are required, binding does not appear to be cooperative. The similarity of the 24p3 promoter with the previously characterized IL-6 promoter prompted us to perform a comparative analysis of numerous IL-17-responsive promoters. We found statistical enrichment of NF-κB and C/EBP sites in addition to phylogenetically conserved AP-1 and Oct-1 sites among these genes.

Exciting new findings indicate that IL-17 is the hallmark of a newly described T cell subset. The prevailing paradigm in immunology indicates that CD4+ T helper cells are divided into two major categories, Th1 and Th2, which are characterized by secretion of interferon γ and IL-4, respectively. Naive Th0 cells are driven to differentiate into these subsets by the actions of specific cytokines; IL-12 drives development of Th1 cells, whereas IL-4 promotes Th2 development. These pathways are self-reinforcing and mutually antagonistic, and therefore, once a T cell begins to differentiate, it becomes committed to this fate. Although this model has explained many observations regarding T cell-mediated host responses, there have been discrepancies related to cytokines that do not fit in either Th subset. Recent studies have identified a subset of CD4+ T cells that secrete IL-17 and the closely related cytokine IL-17F as well as other inflammatory cytokines such as TNFα and IL-6. These Th17 cells arise as a unique T helper subset independently of Th1 and Th2 (3–5, 36, 46–49). Strikingly, Th17 cells are sufficient to drive pathology in experimental autoimmune encephalomyelitis, the mouse model of multiple sclerosis, indicating that functions previously ascribed to Th1 cells may in fact be caused by IL-17 (47, 50, 51). Altogether, much recent data highlights the central importance of IL-17 in immunity and inflammation.

Once expressed, IL-17 functions primarily in the innate arm of the immune system. Most studies have focused on the role of IL-17 as a regulator of neutrophils, largely via control of chemokine expression, but also ICAM-1 and granulocyte-colony stimulating factor (for review, see Refs. 1 and 15). IL-17 receptor-deficient mice are highly susceptible to various bacterial, fungal, and parasitic organisms, as they consistently fail to mount effective neutrophil responses. This is associated with a drastic reduction in expression of neutrophil-attractive chemokines, including Groo/KC (CXCL1), MIP2 (CXCL2), and LIX (CXCL5) (16, 18). In addition, IL-17 and IL-23 have been linked to an important homeostatic control mechanism for neutrophils (52). In addition to the chemokine-neutrophil axis, IL-17 regulates expression of other genes involved in innate immu-
nity, including IL-6, defensins, inflammatory transcription factors, mucins, and acute phase proteins such as 24p3 and complement (19, 29, 30). The relative importance of IL-17 to each of these gene targets in particular in vivo settings has yet to be established.

Despite the importance of IL-17-mediated gene expression in vivo, relatively little data are available regarding its mechanisms of gene regulation. Our data with the 24p3 and IL-6 genes indicate that NF-κB and C/EBP are both essential for IL-17-mediated transcription (Fig. 2 (6)). Although the importance of the NF-κB site has been confirmed for many IL-17-inducible genes, the significance of C/EBP has been largely overlooked. This may be particularly important in light of IL-17 and TNFα synergy. Nearly all IL-17-induced genes that we have examined exhibit additive or synergistic induction when combined with TNFα (6, 19, 53). Several mechanisms of cooperative activity have been proposed, such as cooperative enhancement of mRNA stability and/or activation of the p38 MAPK pathway (37, 38, 40, 54–56). Synergy does not occur at the level of the receptor, as TNFα and IL-17 do not appear to regulate each other’s receptors (data not shown). We previously found that C/EBPβ is essential for IL-17-mediated expression of IL-6. Moreover, overexpression of C/EBPβ or C/EBPβ can substitute for IL-17-induced activation of the IL-6 promoter (6). Similarly, overexpression of C/EBP can also drive 24p3 promoter expression (19). In the present study we showed that IL-17 induces expression and DNA binding of C/EBPβ and C/EBPβ proteins ~2 h after stimulation, which remain elevated for at least 24 h (Fig. 4). Although both TNFα and IL-17 stimulate NF-κB DNA binding strongly in ST2 cells, it was only sustained up to 8 h in response to IL-17, whereas even at 24 h TNFα continued to activate NF-κB DNA binding. Along the same lines, IL-17 also regulates expression of I-κBζ (45), which was recently shown to cooperate in regulation of both IL-6 and the human homologue of 24p3 in response to IL-1β or lipopolysaccharide but not TNFα (57, 58). Therefore, the cooperativity between IL-17 and TNFα may in part be due to their differential activities on two key regulatory TFs, NF-κB and C/EBP, which combine to drive strong, sustained transcription of 24p3. In studies of the human 24p3/neutrophil gelatinase-associated lipocalcin gene in A549 pneumocyte-derived cells, NF-κB was found to be important for IL-1 and TNFα inducibility, but surprisingly C/EBP was not identified as an essential transcription factor. In this study IL-17 was not tested, so this may indicate an underlying difference in how IL-1β signaling differs from IL-17 (59).

To obtain a more comprehensive picture of promoter elements common to IL-17 target genes, we examined predicted and/or validated transcription factor binding sites in the proximal promoters of 18 IL-17-induced genes from both mouse and human. This analysis showed statistical enrichment of four TF binding sequences in IL-17 target promoters, including sites for NF-κB, C/EBP, AP-1, and Oct-1 (Tables 1 and 2). It was not surprising to find that NF-κB sites were overrepresented, since considerable work has been shown that IL-17 activates NF-κB, and this is important for target gene expression (6, 29, 31, 60). The mitogen-activated protein kinase pathway has also been linked to IL-17 in various studies, although neither IL-6 nor 24p3 appears to be regulated by AP1 directly (Ref. 6 and data not shown). Oct-1 is a member of the POU (Pit-1, Oct 1/2, Unc-86) TFs and is widely expressed. It was recently shown to be involved in cellular responses to stress such as γ-irradiation and hydrogen peroxide (61), but its functional role in mediating IL-17 gene expression is as yet unclear.

A new finding from our computational analysis is that IL-17 target promoters fall into three categories based on the relative presence and locations of TFBSs and which correlate well with their biological activities (Table 3, Fig. 5). Group 1 genes include immuno-regulatory molecules such as IL-6 and 24p3 and contain both NF-κB and C/EBP sites in their proximal promoters (defined as within 600 bp of the TSS). Because IL-17 activates C/EBP expression, we predict that this category of genes will be positively amplified by C/EBP and perhaps sustained over relatively long time periods. Group 2 genes have NF-κB sites in their proximal promoters but infrequent C/EBP sites at more distal locations and were uniformly chemokines. In this regard evolutionary relationships between human and murine chemokine genes are complex. For example, the CXCL5 gene in mice is LIX, whereas its counterpart in humans is either GCP-2 or ENA-78 (62) (here, we used ENA-78 for the species alignment, Fig. 5). Although the NF-κB site is highly conserved among all three chemokine promoter regions, a conserved proximal C/EBP site is found in the human promoters but not in LIX (62). Finally, Group 3 genes are regulated independently of NF-κB, and no obvious pattern of TF usage was common to this group. However, it should be noted that C/EBPβ and C/EBPδ have CEBP binding sites in their own regulatory regions (in the case of C/EBPδ, this is located downstream of the coding region, (63)), which could potentially serve as a positive feedback autoregulatory mechanism. In summary, these subgroups may lend insight into different signaling mechanisms used by IL-17 to regulate gene expression and thereby shape immune responses.

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