Functional Cooperation between Interleukin-17 and Tumor Necrosis Factor-α Is Mediated by CCAAT/Enhancer-binding Protein Family Members*

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Interleukin (IL)-17 is a recently described cytokine involved in the amplification of inflammatory responses and pathologies. A hallmark feature of IL-17 is its ability to induce expression of other cytokines and chemokines. In addition, IL-17 potently synergizes with tumor necrosis factor-α (TNFα) to up-regulate expression of many target genes, particularly IL-6. Despite the many observations of IL-17 signaling synergy observed to date, little is known about the molecular mechanisms that underlie this phenomenon. In the osteoblastic cell line MC-3T3, we have found that IL-17 and TNFα exhibit potent synergy in mediating IL-6 secretion. Here, we show that at least part of the functional cooperation between IL-17 and TNFα occurs at the level of IL-6 gene transcription. Both the NF-κB and CCAAT/enhancer-binding protein (C/EBP; NF-IL6) sites in the IL-6 promoter are important for cooperative gene expression, but NF-κB does not appear to be the direct target of the combined signal. Microarray analysis using the Affymetrix mouse MG-U74v2 chip identified C/EBPα as another gene target of combined IL-17- and TNFα-induced signaling. Because C/EBP family members are known to control IL-6, we examined whether enhanced C/EBPα expression is involved in the cooperative up-regulation of IL-6 by IL-17 and TNFα. Accordingly, we show that C/EBPα (or the related transcription factor C/EBPβ) is essential for expression of IL-6. Moreover, overexpression of C/EBPα (and, to a lesser extent, C/EBPβ) could substitute for the IL-17 signal at the level of IL-6 transcription. Thus, C/EBP family members, particularly C/EBPα, appear to be important for the functional cooperation between IL-17 and TNFα.

IL-17 is the founding member of an emerging family of inflammatory cytokines whose functions remain incompletely defined (reviewed in Ref. 1). IL-17 is produced almost exclusively by activated T cells and is found predominantly in the T cell memory compartment (2, 3). In contrast, its receptor is ubiquitously expressed, making almost any cell a potential target of this cytokine (4, 5). IL-17 has been implicated in a number of inflammatory diseases, including rheumatoid arthritis, psoriasis, multiple sclerosis, allergic skin immune responses, and inflammation-induced bone loss (6–11). Furthermore, this cytokine amplifies the immune response by triggering the production of cytokines (IL-6, TNFα, and IL-β), chemokines (RANTES (regulated on activation normal T cell expressed and secreted), MCP-1 (monocyte chemoattractant protein-1), MIP-2/IL-8, and GROs), cell-surface markers (RANKL and ICAM-1 (intercellular adhesion molecule-1)), and pro-inflammatory mediators (prostaglandin E2, nitric oxide, and cyclooxygenase-2) (reviewed in Ref. 1). Thus, it is clear that a major role of IL-17 is to interact with the cytokine network, trigger the release of inflammatory mediators, and thereby provide a link between T cell activation and inflammation.

One of the main IL-17 signaling targets is the cytokine IL-6. Like most cytokines, IL-6 exerts pleiotropic biological effects, including induction of acute-phase proteins, B cell differentiation, and bone turnover (reviewed in Ref. 12). Expression of IL-6 is controlled at many points, particularly at the level of transcription; and consequently, numerous inflammatory agonists induce its expression. Indeed, the IL-6 promoter has been described as a biosensor for environmental stress and is activated by bacterial endotoxins; viruses; cell-surface molecules; hormones; and inflammatory cytokines such as IL-1β, TNFα, and transforming growth factor-β (13–16). IL-6 expression is also negatively controlled by glucocorticoids, estrogen, androgens (17, 18). The receptor signaling pathways leading to IL-6 gene expression result in the activation of several transcription factors, including NF-κB, CCAAT/enhancer-binding protein (C/EBP; NF-IL6), and AP-1 (14, 19, 20).

IL-17 has been shown to induce secretion of IL-6 in a variety of cell backgrounds, including macrophages, fibroblasts, osteoblasts, epithelial cells, and chondrocytes (4, 21–22). However,

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1 The abbreviations used are: IL, interleukin; TNFα, tumor necrosis factor-α; C/EBP, CCAAT enhancer-binding protein; MAPK, mitogen-activated protein kinase; ELISA, enzyme-linked immunosorbent assay; MEFs, murine embryonic fibroblasts; α-MEM, minimum essential medium eagle, α-modification; PBS, fetal bovine serum; EMSA, electrophoretic mobility shift assay; GAPD, glyceraldehyde-3-phosphate dehydrogenase.
the production of IL-6 is dramatically increased when cells are treated with IL-17 together with other pro-inflammatory cytokines, particularly IL-1β and TNFα (2). Importantly, the combination of IL-17 with TNFα and/or IL-1β better reproduces the microenvironment of most inflammatory diseases (e.g. rheumatoid arthritis), where all of these cytokines are present at elevated levels and probably act cooperatively or synergistically (reviewed in Refs. 24 and 25). In humans, antibodies to TNFα or soluble TNF receptor molecules are effective treatments for rheumatoid arthritis (25, 26). Intriguingly, in a mouse model of arthritis, combining TNFα blockade with agents that also block IL-17 could control synovial inflammation and bone resorption without blocking TNFα alone (27). Although numerous studies have addressed how inflammatory agonists work individually to drive IL-6 expression, far less is known about how they function in concert.

IL-17 has been shown to exhibit signaling synergy with other cytokines or agonists in various systems (e.g. Refs. 6 and 28–31), yet the molecular mechanisms responsible for this phenomenon remain unclear. For example, cooperation between IL-17 and interferon-γ has been demonstrated in keratinocytes (6), corneal fibroblasts (32), and pancreatic pericarin myofibroblasts (33). Similarly, IL-17 and CD40L synergistically enhance IL-6 production in renal epithelial cells (34). One partial mechanism of synergy in this system is IL-17-induced up-regulation of CD40 surface expression, yet other mechanisms such as convergence of signaling pathways probably exist. Furthermore, combinations of IL-17 and TNFα were shown to enhance mRNA transcript stability of IL-6, cyclooxygenase-2, and GROα (29, 31, 35), events that are probably dependent on MAPK signaling pathways (35, 36). Thus, cooperation in cytokine signaling clearly occurs at multiple levels.

In this study, we have addressed the mechanism by which IL-17 and TNFα control synergistic expression of IL-6 in osteoblastic bone cells. Using microarray analysis, we identified C/EBPβ (NF-IL6) as a target of the combined action of IL-17 and TNFα and show that this transcription factor appears to be involved in mediating cooperative transcriptional activation of the IL-6 gene by these cytokines.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Reagents, Stimulations, and ELISAs**—Mouse calvarial-derived osteoblastic MC-3T3-E1 cells, wild-type MEFs (kindly provided by Dr. Wen-Chen Yeh), and C/EBPβ/−/− MEFs were cultured in α-MEM (Sigma) supplemented with 10% heat-inactivated FBS (Gemini Bioproducts, Woodland, CA), penicillin, streptomycin, and L-glutamine (Invitrogen). Recombinant human IL-17 and TNFα were obtained from R&D Systems (Minneapolis, MN). For stimulations, cells were seeded at 1 × 10⁶ cells/ml in α-MEM and 10% FBS. Following attachment, cells were washed twice with phosphate-buffered saline, incubated in α-MEM and 0.3% FBS overnight, and stimulated with the indicated cytokines for the designated time periods. Supernatants were analyzed in triplicate for IL-6 by sandwich ELISA (eBioscience and Pharmingen) according to the manufacturers’ instructions.

**Microarray Analysis**—MC-3T3 cells were grown to confluence in α-MEM (Sigma) supplemented with 10% heat-inactivated FBS (Gemini Bioproducts, Woodland, CA), penicillin, streptomycin, and L-glutamine (Invitrogen). Recombinant human IL-17 and TNFα were obtained from R&D Systems (Minneapolis, MN). For stimulations, cells were seeded at 1 × 10⁶ cells/ml in α-MEM and 10% FBS. Following attachment, cells were washed twice with phosphate-buffered saline, incubated in α-MEM and 0.3% FBS overnight, and stimulated with the indicated cytokines for the designated time periods. Supernatants were analyzed in triplicate for IL-6 by sandwich ELISA (eBioscience and Pharmingen) according to the manufacturers’ instructions.

**Northern, Western, and EMSA Analyses**—For Northern blotting, MC-3T3 cells or MEFs were incubated for 24 h in α-MEM and 0.3% FBS and stimulated for 2 h with the indicated cytokines. Total RNA was prepared using the RNeasy kit. RNA (10 μg/sample) was separated on a 1.4% denaturing formaldehyde-agarose gel; transferred to nylon membrane (Zeta-Probe, Bio-Rad); and probed with 32P-labeled cDNA probes corresponding to C/EBPβ (kindly provided by Dr. L. Vales). murine IL-6 (kindly provided by Dr. Heinz Baumann), and GAPD (American Type Culture Collection, Manassas, VA). Probes were labeled using the MegaPrime labeling system (Amersham Biosciences). For Western blotting, cells were stimulated as described above, and nuclear extracts were prepared as described previously (37, 38). Samples of nuclear extracts normalized to equal concentrations (20–40 μg/sample) were boiled in SDS sample buffer, separated on 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and blotted with antibodies to C/EBPβ (sc-151, Santa Cruz Biotechnology, Santa Cruz, CA) or α-tubulin (TU-01, Zymed Laboratories Inc., South San Francisco, CA).

**RESULTS**

**IL-17 Synergizes with TNFα to Induce IL-6 Secretion**—Similar to findings in other cell types (4), we found that IL-17 induced the production of IL-6 in MC-3T3 cells in a dose-dependent manner following a 24-h stimulation (Fig. 1A). Surprisingly, very high levels of IL-17 (400–500 ng/ml) were needed to trigger maximal IL-17-induced IL-6 secretion. However, in the presence of low serum levels (0.3% FBS), even the highest amount of IL-6 secretion was still quite minimal, particularly in contrast, IL-17 triggered at least 5-fold higher levels of IL-6 in osteoblastic MC-3T3-E1 cells, wild-type MEFs (kindly provided by Dr. W. Eickelberg (14), and the (NF-IL6)−/− luciferase reporter was provided by Dr. Xin Lin. Where indicated, cells were cotransfected with a 10-fold excess of a control vector (pCMV4) (39) or PCMV5-C/EBPβ or pCMV5-C/EBPβ (generously provided by Dr. Lynn Vales (40). In all cases, 10 ng of the Renilla luciferase plasmid (kindly provided by Dr. Xin Lin) was cotransfected as an internal standard. Cells were then stimulated with the indicated cytokines for either 6 h (with the (NF-IL6)−/− luciferase reporter) or 24 h (with the IL-6-luciferase reporter) and lysed, and supernatants were analyzed for luciferase activity using an Orion MPl2 luminometer (Berthold Detection Systems, Oak Ridge, TN). For reconstitution of C/EBPβ/−/− MEFs, 0.5 × 10⁷ cells were transfected with 2 μg of the indicated plasmids in LipofectAMINE, and supernatants were analyzed for IL-6 as described above.

Although a relatively high concentration of IL-17 (200 ng/ml) was insufficient to induce dramatic IL-6 secretion under low serum conditions (Fig. 1A), we found that this concentration of IL-17 could potently synergize with a suboptimal dose of TNFα (Fig. 1, A and B). Note that the term synergy is defined as an effect greater than the sum of the that observed with either cytokine alone. It should also be noted that “suboptimal” con-
Enhancement of IL-6 transcription does not entirely account for the cooperative effects of these cytokines in regulating IL-6 expression.

The IL-6 promoter contains binding sites for several transcription factors previously identified to be important for its regulation, including AP-1, C/EBP, and NF-κB (Ref. 14 and references therein). Using promoter constructs specifically mutated at these sites, we found that the AP-1 site was not necessary for IL-17/TNFα-induced promoter activity (Fig. 2A and Table I). Note that the slight enhancement of overall reporter activity with the pIL-6-Luc651ΔAP-1 construct was not routinely observed. In contrast, in the IL-6 promoters containing mutations at the C/EBP or NF-κB sites, the overall magnitude of stimulation by IL-17, TNFα, or both cytokines together was greatly reduced. Interestingly, however, the constructs were still slightly stimulated by IL-17 or TNFα alone, and the cytokines together enhanced promoter activity still further (Table I). Thus, the NF-κB and C/EBP transcription factors appear to play important roles in regulating IL-6 gene expression, and they are also important, although perhaps not sufficient, for the cooperative action of IL-17 and TNFα.

Because the IL-6 promoter contains a binding site for NF-κB that is important for its transcription (19) and because both IL-17 and TNFα have been reported to activate the NF-κB signaling pathway (4, 41), we assessed whether IL-17 and TNFα together promote any increase in NF-κB activity by using a reporter construct containing five tandem NF-κB consensus sites cloned upstream of luciferase. IL-17 induced only minimal reporter gene activity with this construct, whereas TNFα triggered a much more marked enhancement of luciferase activity (Fig. 2B). Similarly, IL-17 and TNFα together did not show any enhancement of luciferase activity compared with TNFα alone at any concentration of IL-17 or TNFα used (Fig. 2B) (data not shown). We also examined the DNA-binding activity of NF-κB in MC-3T3 cells by EMSA using a probe encompassing the NF-κB site from the IL-6 promoter (Fig. 2C). IL-17 alone induced no detectable NF-κB-DNA binding in MC-3T3 cells even at very high concentrations of cytokine (Fig. 2C, second lane) (data not shown), whereas even low levels of TNFα (2 ng/ml) triggered substantial binding to the NF-κB probe (third lane). This binding was specific, as it did not occur when a mutant version of the NF-κB site was used (seventh through tenth lanes) and was competed specifically with an excess of unlabeled oligonucleotide probe (data not shown). The band was further confirmed to contain NF-κB by supershifting the complex with an antibody to the p65 subunit of NF-κB (sixth lane). IL-17 and TNFα together did not promote significant enhancement of NF-κB-DNA binding compared with TNFα alone. (Note that, although the fourth lane shows a slight increase in DNA-binding activity, this was not a reproducible finding (data not shown).) Therefore, IL-17 and TNFα act in an additive manner to activate the IL-6 promoter. However, although both the C/EBP and NF-κB sites appear to be important for IL-6 gene expression, the direct target of functional cooperation does not appear to be NF-κB itself.

IL-17 and TNFα Regulate IL-6 via C/EBPα—To define further the molecular mechanism by which IL-17 and TNFα cooperate, we used microarrays to assess differences in gene expression in MC-3T3 cells stimulated with suboptimal concentrations of TNFα alone compared with TNFα and IL-17 together. We examined the shortest time point at which synergistic signaling leading to IL-6 production was apparent, viz., 2 h post-stimulation (Fig. 1B). Synergistic signaling was confirmed by showing that there was a 5-fold increase in IL-6 secreted from the IL-17/TNFα-stimulated samples compared with the TNFα-stimulated samples (Fig. 3A). Affymetrix microarray analysis

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Fig. 1. IL-17 and TNFα synergize to induce IL-6 in MC-3T3 cells. A, MC-3T3 cells were incubated in α-MEM and 0.3% FBS together with the indicated concentrations of IL-17 for 24 h. In the last sample, cells were stimulated with 200 ng/ml IL-17 and a suboptimal dose of TNFα (7 ng/ml). Supernatants were analyzed in triplicate for the presence of murine IL-6 by sandwich ELISA, and S.D. values are shown. B, MC-3T3 cells were incubated in α-MEM and 0.3% FBS and left unstimulated (Unstim.; white bars) or stimulated with 200 ng/ml IL-17 (gray bars), 2 ng/ml TNFα (black bars), or IL-17 (200 ng/ml) and TNFα (2 ng/ml) (hatched bars) for the indicated time periods. Supernatants were analyzed in triplicate for the presence of IL-6 as described for A.

Centrations of TNFα were determined empirically for each batch of cytokine and ranged between 0.02 and 2 ng/ml (Fig. 1B) (data not shown). Importantly, IL-17 did not trigger secretion of endogenous TNFα in these cells, and the medium alone did not contain residual IL-6. This functional cooperation between IL-17 and TNFα signaling occurred rapidly, with synergistic levels of IL-6 produced as early as 2 h post-stimulation (Fig. 1B).

Functional Cooperation between IL-17 and TNFα Occurs Partially at the Level of IL-6 Transcription—Although signaling synergy between IL-17 and TNFα has been reported previously (6, 28–32), the mechanisms that underlie this phenomenon remain poorly understood. To determine whether these cytokines function synergistically or additively at the level of IL-6 gene transcription, we transfected a 651-bp region of the IL-6 promoter fused to the luciferase reporter gene (14) into MC-3T3 cells and measured luciferase activity in response to IL-17, TNFα, or both cytokines together (Fig. 2A). Both IL-17 (200 ng/ml) and suboptimal levels of TNFα stimulated detectable reporter gene activity, whereas both cytokines together triggered an approximately additive increase in activity. This finding was highly reproducible and indicates that, although enhancement of IL-6 transcription does not entirely account for the signaling synergy between IL-17 and TNFα, it is at least partly responsible for the cooperative effects of these cytokines in regulating IL-6 expression.

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2 M. J. Ruddy, unpublished data.
Functional cooperation between IL-17 and TNFα lead-
ing to IL-6 expression occurs partly at the level of IL-6 gene transcription. A, MC-3T3 cells were transfected in triplicate with luciferase reporter constructs containing the wild-type IL-6 promoter or its mutants and incubated with IL-17 (200 ng/ml), TNFα (2 ng/ml), or both cytokines together as described in the legend to Fig. 1. Six hours later, cellular lysates were analyzed for luciferase activity. Results were nor-
malized to an internal Renilla luciferase control, and S.D. values are shown. B, MC-3T3 cells were transfected in triplicate with a reporter construct containing five tandem NF-κB sites upstream of luciferase; and 6 h later, cellular lysates were analyzed for luciferase activity. Results were normalized to an internal Renilla luciferase control, and S.D. values are shown. C, MC-3T3 cells were stimulated for 2 h with IL-17 (17; 200 ng/ml) and/or TNFα (T, 2 ng/ml), and nuclear extracts from cytokine-stimulated MC-3T3 cells were subjected to SDS-PAGE, transferred to nitrocellulose, and blotted with antibodies to murine C/EBPβ or α-tubulin as a loading control (Fig. 4C). To quantitate changes in protein levels, Western blots from two independent experiments were subjected to scanning densitometry, and the average increases in band intensities are shown (Fig. 4C) (data not shown). Similar to our observations of C/EBPβ mRNA regulation, the C/EBPβ protein was then used to identify genes up- or down-regulated under these conditions, with the aim of revealing genes potentially involved in mediating cooperative/synergistic signaling or identifying other genes whose promoters might be similarly controlled. The experiment was performed with separately prepared samples on two different occasions, with highly similar results (Fig. 3). Several genes already known to be regulated by IL-17 and TNFα were found to be enhanced, including IL-6 and the chemokine GRO1 (Fig. 3B) (data not shown) (31). In addition, the chemokine RANTES/ScyA5 was up-regulated by this cytokine combination, which contrasts with other cell back-
grounds in which IL-17 has been found to down-regulate RANTES/ScyA5 expression (32, 42). Strikingly, in both experi-
ments, C/EBPβ was enhanced by an average of 3.4-fold in the IL-17/TNFα-treated cells compared with the TNFα-treated cells (Fig. 3B). It is noteworthy that C/EBPβ (or any other C/EBP isoform) was not enhanced under these conditions, even though this factor is also thought to be important in regulating IL-6 expression under many circumstances (reviewed in Ref. 43 and see “Discussion”).

To examine the profile of C/EBPβ gene regulation in more detail, we performed Northern blot analyses of mRNA prepared from MC-3T3 cells stimulated with TNFα and/or IL-17. C/EBPβ mRNA was slightly enhanced by either IL-17 or TNFα alone, but was induced even more strongly after IL-17/TNFα co-stimulation. As with IL-6 mRNA, C/EBPβ mRNA was enhanced approximately additively (or slightly synergistically) by IL-17 and TNFα compared with either cytokine alone as determined by scanning densitometry (Fig. 4A). Because IL-17 and/or TNFα stimulation of MC-3T3 cells results in the release of IL-6 and other cytokines and chemokines that have also been implicated in enhancing C/EBPβ (43), we used cycloheximide to block the production of proteins that might indirectly cause C/EBPβ to be expressed. As shown, cycloheximide treatment did not reproducibly inhibit the up-regulation of C/EBPβ mRNA (Fig. 4A), although it completely blocked production of IL-6 from the same cells (Fig. 4B). Similarly, a neutralizing antibody to IL-6 did not inhibit the up-regulation of C/EBPβ mRNA (data not shown). To confirm that C/EBPβ protein levels were also cooperatively enhanced by IL-17 and TNFα, nuclear extracts from cytokine-stimulated MC-3T3 cells were subjected to SDS-PAGE, transferred to nitrocellulose, and blotted with antibodies to murine C/EBPβ or α-tubulin as a loading control (Fig. 4C). To quantitate changes in protein levels, Western blots from two independent experiments were subjected to scanning densitometry, and the average increases in band intensities are shown (Fig. 4C) (data not shown). Similar to our observations of C/EBPβ mRNA regulation, the C/EBPβ protein mRNA regulation, the C/EBPβ mRNA was slightly enhanced by either IL-17 or TNFα alone, but was induced even more strongly after IL-17/TNFα co-stimulation. As with IL-6 mRNA, C/EBPβ mRNA was enhanced approximately additively (or slightly synergistically) by IL-17 and TNFα compared with either cytokine alone as determined by scanning densitometry (Fig. 4A). Because IL-17 and/or TNFα stimulation of MC-3T3 cells results in the release of IL-6 and other cytokines and chemokines that have also been implicated in enhancing C/EBPβ (43), we used cycloheximide to block the production of proteins that might indirectly cause C/EBPβ to be expressed. As shown, cycloheximide treatment did not reproducibly inhibit the up-regulation of C/EBPβ mRNA (Fig. 4A), although it completely blocked production of IL-6 from the same cells (Fig. 4B). Similarly, a neutralizing antibody to IL-6 did not inhibit the up-regulation of C/EBPβ mRNA (data not shown). To confirm that C/EBPβ protein levels were also cooperatively enhanced by IL-17 and TNFα, nuclear extracts from cytokine-stimulated MC-3T3 cells were subjected to SDS-PAGE, transferred to nitrocellulose, and blotted with antibodies to murine C/EBPβ or α-tubulin as a loading control (Fig. 4C). To quantitate changes in protein levels, Western blots from two independent experiments were subjected to scanning densitometry, and the average increases in band intensities are shown (Fig. 4C) (data not shown). Similar to our observations of C/EBPβ mRNA regulation, the C/EBPβ protein
IL-17 and TNFα Regulate IL-6 via C/EBPβ

Table I
- Fold increases in normalized luciferase activity values from Fig. 2A

|                      | pIL-6-Luc651 | pIL-6-Luc651AP-1 | pIL-6-Luc651C/EBP | pIL-6-Luc651ΔNF-xB |
|----------------------|--------------|------------------|-------------------|-------------------|
| Unstimulated        | 1.0          | 1.0              | 1.0               | 1.0               |
| IL-17               | 1.9          | 2.2              | 1.7               | 1.4               |
| TNFα                | 2.0          | 2.4              | 1.5               | 1.2               |
| IL-17 + TNFα        | 4.0          | 4.3              | 2.1               | 1.9               |

Fig. 3. C/EBPβ is up-regulated by IL-17 and TNFα in Affymetrix™ microarrays. A, supernatants from MC-3T3 cells stimulated with TNFα (2 ng/ml) alone or with IL-17 (200 ng/ml) were analyzed for IL-6 by ELISA as described in the legend to Fig. 1. B, shown are the fold increases in genes induced in IL-7- and TNFα-induced samples compared with TNFα-induced samples as determined by Affymetrix analysis. The average increases from duplicate experiments are shown.

was enhanced approximately additively by IL-17 and TNFα co-stimulation. Thus, the cytokine-induced changes in C/EBPβ parallel changes in IL-6 gene expression. Together, these data indicate that IL-17 and TNFα directly induce the up-regulation of C/EBPβ in MC-3T3 cells, which could potentially play a role in the induction of IL-6 gene expression.

Either C/EBPβ or C/EBPβ Is Necessary for IL-6 Production—To determine whether C/EBPβ is necessary for IL-6 production, a doubly deficient C/EBPβ−/−/C/EBPβ−/− MEF cell line (termed C/EBPβ−/− MEF) (44) was transiently transfected with either a control plasmid or an expression vector encoding C/EBPβ or C/EBPβ. Following transfection, cells were stimulated with IL-17 and/or TNFα for 24 h, and an IL-6 ELISA was performed on cell supernatants. As expected and consistent with Fig. 2A, C/EBPβ−/− MEFs transfected with a control vector did not produce detectable IL-6 following cytokine stimulation. However, cells that were reconstituted with C/EBPβ showed IL-6 secretion after IL-17 and TNFα co-stimulation, indicating that functional cooperation can be restored in the presence of C/EBPβ. Interestingly, cells transfected with C/EBPβ also showed expression of IL-6 following IL-17 and TNFα co-stimulation at a somewhat higher level than the C/EBPβ-transfected cells. Therefore, reconstitution of C/EBPβ proteins promotes IL-17- and TNFα-induced IL-6 production, and C/EBPβ and C/EBPβ are functionally interchangeable in this regard. Furthermore, there are apparently no other C/EBP isoforms in MC-3T3 cells that can substitute for C/EBPβ and C/EBPβ, as the control transfected cells did not induce detectable IL-6. Consistent with these data, we found that C/EBPβ−/− MEFs also failed to induce IL-6 mRNA or protein following cytokine stimulation (Fig. 5, B and C), whereas wild-type MEFs showed enhanced IL-6 secretion in response to these cytokines (Fig. 5A). Interestingly, the degree of functional cooperation between these cytokines in MEFs was considerably less marked than in MC-3T3 cells. A consistent explanation for this observation is that C/EBPβ is expressed at a comparatively high basal level in wild-type MEFs compared with MC-3T3 cells (Fig. 5, B and C) and is not as strikingly up-regulated by IL-17 and TNFα co-stimulation as in MC-3T3 cells.

C/EBP Overexpression Can Substitute for IL-17 Signaling—Finally, we sought to determine whether C/EBPβ and/or C/EBPβ is responsible for the functional cooperation of IL-17 and TNFα at the level of the IL-6 promoter. To this end, MC-3T3 cells were transfected with the IL-6-luciferase reporter gene together with either a control vector or a 10-fold excess of C/EBPβ or C/EBPβ. Cells were then left untreated or were stimulated with IL-17, TNFα, or both cytokines together, and lysates were assayed for reporter gene activity 24 h later. As previously observed, cells transfected with the control vector displayed an approximately additive induction of IL-6 promoter activity following IL-17 and TNFα co-stimulation. In contrast, cells transfected with C/EBPβ and stimulated with TNFα alone (but not IL-17 alone) displayed significantly elevated promoter activity. Indeed, in cells transfected with the control vector, luciferase activity stimulated by TNFα was essentially the same as that induced by IL-17, whereas in cells transfected with C/EBPβ, the activity in the TNFα-stimulated sample was consistently 1.7-fold higher than that in the IL-17-stimulated sample. Interestingly, transfection of C/EBPβ also enhanced TNFα-dependent IL-6 promoter activity, and the TNFα-induced signal was also 1.7-fold higher than in the sample stimulated with IL-17 alone. In contrast, overexpression of the p65 subunit of NF-κB did not exhibit this enhancing effect on the IL-17 or TNFα signal (data not shown). This result suggests that C/EBP family members can substitute for IL-17 in enhancing IL-6 promoter activity in the presence of TNFα and...
are therefore likely a significant target of the cooperative signal induced by IL-17 and TNFα.

**DISCUSSION**

In this study, we have examined the molecular mechanism by which two inflammatory cytokines (IL-17 and TNFα) function in concert to direct synergistic expression of IL-6. At least part of the functional cooperation leading to IL-6 expression occurs at the level of gene transcription, and the transcription factor-binding sites for NF-κB and C/EBP within the IL-6 promoter appear to both be involved in mediating the combined effects of IL-17 and TNFα. Microarray analyses identified C/EBPδ as another gene target of IL-17 and TNFα cooperative signaling. Because C/EBP family members in turn regulate IL-6, we determined whether enhanced C/EBPδ expression was involved in the combined signal. Indeed, C/EBPδ (or the related
transcription factor C/EBPβ is necessary for the cooperative expression of IL-6 in MEFs, and overexpression of C/EBPβ or C/EBPδ can at least partly replace the IL-17 signal at the level of IL-6 transcription. The IL-6 promoter has long been recognized to contain a pivotal C/EBP-binding site, located −150 bp upstream of the transcriptional start site (45); and we have shown that this site is indeed important, if not essential, for the combined signaling between IL-17 and TNFα (Fig. 2). Recently, several other potential C/EBP sites were identified in the vicinity of the NF-κB site, and these sites have been shown to bind C/EBP family members in overexpression systems (40). However, we have been unable to detect binding of any C/EBP isoforms to this region in nuclear extracts taken from IL-17- and TNFα-stimulated MC-3T3 cells (Fig. 2) (data not shown), suggesting that these sites are less important to IL-6 gene regulation in osteoblasts. In addition, the transcriptional repressor protein RBP (CBF1) has been shown to bind to a region overlapping the NF-κB site (46), but we could not demonstrate recombination signal sequence binding protein binding by EMSA, and RBP gene expression was not altered in response to IL-17 and/or TNFα (data not shown).

C/EBPβ is a member of the CCAAT enhancer-binding protein family (reviewed in Ref. 43), which contains transcription factors that are characterized by a basic leucine zipper motif and

Fig. 6. C/EBPβ and C/EBPδ can substitute for IL-7 signaling in mediating IL-6 production. MC-3T3 cells were transiently transfected with the IL-6-luciferase reporter gene as described in the legend to Fig. 2, together with a 10-fold excess of C/EBPδ, C/EBPβ, or control (pCMV4) expression vector. Note that, in this experiment, the IL-6 expression vector with a mutant AP-1 site was used, but similar results were obtained using the wild-type parental IL-6 promoter construct (X. K. Liu, unpublished data). Unstim., unstimulated.
that play central roles in diverse physiological events. They bind to very similar promoter elements, located in a wide variety of gene targets. C/EBPα was reported to be expressed constitutively in osteoblasts, where it regulates the insulin-like growth factor gene (47). In MC-3T3 cells, however, C/EBPδ appears to be present at a relatively low basal level, but is strongly inducible at the mRNA and protein levels by inflammatory cytokines (Fig. 4). In the case of IL-17- and TNFα-induced co-stimulation of C/EBPδ, the signal appears to be direct because treatment with cycloheximide or anti-IL-6 antibody did not prevent the appearance of C/EBPδ mRNA (Fig. 4). At present, we do not know the nature of the IL-17- and TNFα-mediated signal that leads to C/EBPδ expression. In this regard, STAT-3 (signal transducers and activators of transcription-3) and C/EBPδ itself have been shown to be involved in its transcriptional regulation, suggesting the possibility of a positive feedback loop leading to C/EBPδ autoregulation; however, much remains to be defined about the details of the genetic control of C/EBPδ (48, 49). Unlike C/EBPδ, C/EBPβ is thought to be controlled mainly at the post-translational level by both phosphorylation and subcellular localization (reviewed in Ref. 43). Consequently, it is not surprising that we did not observe dramatic enhancement of C/EBPβ mRNA following IL-17 and TNFα stimulation (Fig. 3), yet C/EBPδ could clearly substitute for C/EBPβ in regulating IL-6 (Figs. 5 and 6).

The IL-6 promoter also contains an NF-κB site, which is important for cooperative signaling by IL-17 and TNFα (Fig. 2). However, we found that IL-17 activated only extremely weak NF-κB nuclear import, DNA binding, or transcriptional activity on a linked reporter gene in MC-3T3 cells (Fig. 2) (data not shown). This finding is consistent with other reports in which IL-17 has been shown to be a relatively poor activator of NF-κB (21, 29). Thus, we hypothesize that, in the context of IL-6 gene expression, NF-κB is controlled primarily by the TNFα-induced signal, whereas cooperative signaling is mediated by C/EBPδ.

Although much is known about TNFα signal transduction (reviewed in Ref. 41), the mechanism of IL-17 receptor signaling is still quite poorly defined (reviewed in Ref. 1). Although we have shown that IL-17 is a poor activator of NF-κB activity in MC-3T3 cells, other pathways could potentially be involved. For example, many of the MAPK pathways have also been shown (reviewed in Ref. 43), to bind to very similar promoter elements, located in a wide variety of gene targets. C/EBPα and β operate at all of these levels, including enhancing mRNA stability (29, 31, 35) and the causing the up-regulation of other cell-surface receptors (31). Here, we show that IL-17 collaborates with TNFα by up-regulating expression of C/EBPα, a central transcription factor involved in IL-6 gene expression. Understanding the molecular targets involved in cytokine synergy may pave the way for the development of improved therapeutic treatments for inflammatory diseases involving IL-17 and other inflammatory cytokines, such as rheumatoid arthritis.

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