NF-κB1 (p50) Homodimers Differentially Regulate Pro- and Anti-inflammatory Cytokines in Macrophages*S

Shanjun Cao, Xia Zhang, Justin P. Edwards, and David M. Mosser1

From the Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, Maryland 20742

Macrophages are on the first line of host defense. In response to infections with microbes or exposure to pathogen-derived products, these cells undergo a series of physiological changes that can collectively be called an activation response. Activated macrophages are more adept at killing intracellular pathogens, and they exhibit dramatic alterations in both the quantity and quality of secreted products. These cells are an important source of inflammatory cytokines, and the production of these cytokines is an important component of host defense (1). The dysregulated overproduction of these cytokines, however, can contribute to autoimmune pathologies, such as those seen in rheumatoid arthritis, inflammatory bowel disease, and multiple sclerosis (2).

One of the ways that macrophages control the overproduction of inflammatory cytokines is to produce the anti-inflammatory cytokine, IL-10.2 IL-10 is a Class II α-helical cytokine, and the founding member of a growing family of structurally related cytokines, including IL-19, IL-20, IL-22, IL-24, and IL-26 (3). IL-10 has well documented immune inhibitory capacity. IL-10 was originally called "cytokine synthesis inhibiting factor," because it can inhibit the transcription and translation of a variety of inflammatory cytokines (4). IL-10 reduces antigen presentation and either inhibits (5) or biases (6) T-cell activation. The administration of exogenous IL-10 to macrophages can render them refractory to interferon-γ (7) and diminish their responses to LPS (8). The well described immunomodulatory activities of IL-10 have resulted in a number of clinical trials using recombinant cytokine to ameliorate autoimmune or inflammatory diseases, including inflammatory bowel disease, rheumatoid arthritis, psoriasis, etc. (9). Several pathogens, including Leishmania (10), Vaccinia virus (11), and Mycobacteria (12), exploit the immunosuppressive activity of IL-10 to establish infection.

One of the paradoxes about the regulation of IL-10 production by macrophages is that the stimuli used to induce inflammatory cytokine production are often the same stimuli that induce IL-10. Only recently have studies begun to reveal how these divergent families of cytokines can be produced in response to similar stimuli. Recent studies have demonstrated that the control of IL-10 biosynthesis depends not only on the activation of transcription factors, but also on covalent modifications to the histones associated with the IL-10 promoter. These modifications render the IL-10 promoter accessible to the transcription factors that bind there (13). Among the several transcription factors that have been implicated in IL-10 transcription, Sp1 appears to play a dominant role (14), and several C/EBP binding sites have also been identified (15). Stat3 (16), c-Maf (17), and NF-Y transcription factors (18) have also been shown to bind to the human IL-10 promoter. To date, there has been no definitive evidence to support a role for NF-κB in the transcriptional control of IL-10 biosynthesis, although its role in the regulation of pro-inflammatory cytokines is well recognized (19).

NF-κB is a family of transcription factors that includes RelA (p65), NF-κB1 (p50 and p105), NF-κB2 (p52 and p100), c-Rel, and RelB. These transcription factors are sequestered in the cytoplasm by inhibitory IKB proteins and are released from these inhibitory interactions following cellular stimuli (20). Upon binding to DNA, NF-κB can form a series of homo- and heterodimers that activate a variety of genes. In order to fully understand the transcriptional activity of NF-κB, it is important to understand the role of different subunits in the regulation of these genes.

NF-κB/Rel is a family of transcription factors whose activation has long been linked to the production of inflammatory cytokines. Here, we studied NF-κB signaling in the regulation of the anti-inflammatory cytokine, interleukin-10 (IL-10). We identified a role for a single NF-κB family member, NF-κB1 (p50), in promoting the transcription of IL-10. The NF-κB cis-element on IL-10 proximal promoter was located to −55/−46, where p50 can homodimerize and form a complex with the transcriptional co-activator CREB-binding protein to activate transcription. The other Rel family members appear to play a negligible role in IL-10 transcription. Mice lacking p50 were more susceptible to lethal endotoxemia, and macrophages taken from these mice exhibit skewed cytokine responses to lipopolysaccharide, characterized by decreased IL-10 and increased tumor necrosis factor and IL-12. Taken together, our studies demonstrate that NF-κB1 (p50) homodimers can be transcriptional activators of IL-10. The reciprocal regulation of pro- and anti-inflammatory cytokine production by NF-κB1 (p50) may provide potential new ways to manipulate the innate immune response.

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1 To whom correspondence should be addressed: Dept. of Cell Biology and Molecular Genetics, University of Maryland, 1103 Microbiology Bldg., College Park, MD 20742. Tel.: 301-314-2594; Fax: 301-314-9489; E-mail: dmoss@umd.edu.

2 The abbreviations used are: IL, interleukin; Bcl-3, B-cell CLL/lymphoma 3; BMMΦ, bone marrow-derived macrophage; CBP, CREB-binding protein; CREB, cAMP-response element-binding protein; C/EBP, CAAT/enhancer-binding protein; ChIP, chromatin immunoprecipitation; EMSA, electrophoresis mobility shift assay; p105sr, p105-based NF-κB super suppressor; p300, E1A-binding protein p300; LPS, lipopolysaccharide; ELISA, enzyme-linked immunosorbent assay; TNF, tumor necrosis factor.
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cytoplasm by IκBs, which prevent NF-κB activation (20), and inhibit nuclear accumulation (21). The degradation of IκBs facilitates the migration of NF-κB into the nucleus, where they typically form heterodimers that bind to the promoters of many immune response genes and activate transcription (reviewed in Ref. 19). Prototypical activators of NF-κB are the ligands for the toll-like receptors, which have been well described and studied (reviewed in Ref. 22). The NF-κB/Rel family members RelA, RelB, and c-Rel have a C-terminal transactivation domain. In contrast to these members, p50 lacks a transactivation domain and, therefore, usually forms a heterodimer to be transcriptionally active (19, 23). p50 homodimers, which retain their ability to bind to NF-κB sites, are thought to be transcriptional repressors (24). However, in vitro studies have shown that p50 can associate with other transcriptional activators, such as Bcl-3 (25) or p300 (26), to activate transcription. A number of immune response genes have NF-κB binding element(s) in their promoters, and in fact the constitutive activation of NF-κB pathways is associated with a variety of inflammatory diseases, such as rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease (23). Recent studies have suggested that the mitogen-activated protein kinase p38 may cooperate with NF-κB and help to determine the specificity of NF-κB-driven gene expression (27). In the present study we examine the role of NF-κB in driving IL-10 transcription and highlight the importance of a single Rel family member, p50, in promoting the production of IL-10.

EXPERIMENTAL PROCEDURES

Animal Care and Use—Normal BALB/c mice were used for the isolation of bone marrow-derived macrophages (BMMs). They were purchased from Takonic (Germantown, NY) and used at 6–8 weeks. NF-κB1 (p50) knock-out mice (B6;129P2-Nfκb1tm1Balj) and their wild-type control mice; Bcl-3 knock-out mice (129:FVB-Bcl3tm1Vep) and their controls were purchased from The Jackson Laboratories (Bar Harbor, ME). All mice were maintained in high-efficiency particulate air (HEPA)-filtered Thoren units (Thoren Caging System) at the University of Maryland (College Park, MD). All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Maryland.

Plasmids—The murine IL-10 promoter-driven luciferase reporter constructs pIL-10(-1538/+64)-luc and pIL-10(-158/+64)-luc were described previously (14). The pIL-10(-82/+64)-luc and its derived mutation constructs were constructed by overlapping PCR (28). The NF-κB consensus tetramer plasmid p(4×NF-κB)-luc was purchased from Clontech Laboratories (Palo Alto, CA). pIL-12p40/3.3kb-luc and pIL-12p35–luc were previously described (29), pTNF-α-luc was generously provided by Dr. Peter F. Johnson (NCI-Frederick, National Institutes of Health) (30). The NF-κB1(p50), RelA (p65), and c-Rel expression constructs were described previously (31), and NF-κBp52 and RelB expression constructs were generously provided by Dr. Hsiou-Chi Liou (Weill Medical College of Cornell University, New York). The adenovirus E1a wild-type expression construct was described previously (32) and generously provided by Dr. Robert J. Kelm (The Ohio State University, Columbus, OH). The CBP expression construct was previously described (33). The p105-based NF-κB super repressor-p105sr was generously provided by Dr. Li Lin (Johns Hopkins University School of Medicine, Baltimore, MD) (34). The p300 expression construct (35) was generously provided by Dr. Kenneth Wu (University of Texas Health Science Center, Houston, TX). The murine Bcl-3 (GenBank™ NM_033601) was amplified by reverse transcription-PCR and cloned into pCDNA3.1 vector and sequenced.

Cytokines and Reagents—LPS from *Escherichia coli* serotype O127:B8 was purchased from Sigma. Ultrapure LPS from *E. coli* 0111:B4, and Pam2CSK4, and Pam3CSK4 were purchased from Invitrogen. Recombinant mouse interferon-γ was purchased from R&D Systems (Minneapolis, MN). Recombinant human NF-κBp50 protein was purchased from Promega (Madison, WI).

Cells and Cell Culture—The macrophage-like RAW 264.7 cell line was obtained from ATCC (Manassas, VA). Primary bone marrow-derived macrophages were generated as previously described (6). Transient transfections in bone marrow-derived macrophages was accomplished by the nucleofection technique (Amaza Biosystems, Gaithersburg, MD), as previously described (13), using the cell line kit T and the U20 program. A pmaxGFP vector was used to monitor the transfection efficiency by flow cytometry. 24 h after transfection, transfected cells were treated with 10 ng/ml LPS for 16 h at which time the supernatants were collected and analyzed by ELISA. RAW 264.7 cell transient transfection and luciferase assays were performed as described before (17).

ELISA—Cytokine secretion by stimulated macrophages was determined by ELISA as previously described (36) using the following antibody pairs: IL-12p40/70, C15.6 and C17.8; IL-10, JES5–2A5 and JES5–16E3; and TNF-α, G281–2626 and MXT3. All antibodies were purchased from BD Biosciences Pharmingen.

Nuclear Extraction and Western Blotting—Nuclear extracts were prepared from stimulated macrophages as described previously (37). For immunoprecipitations, 800 μg of nuclear extract was isolated from 2-h LPS-treated RAW264.7 cells. Extracts were pre-cleared by the addition of 1 μg of rabbit IgG and protein G plus–agarose preincubated with 10% fetal bovine serum. The pre-cleared extracts were then incubated with rabbit anti-CBP antibody or normal rabbit IgG and rotated overnight at 4°C. Bound proteins were eluted by using 1× SDS-PAGE buffer, and the precipitated proteins were analyzed by Western blotting.

In Vivo Responses to Endotoxin—To determine endotoxin sensitivity, both the NF-κB1 knock-out and wild-type control mice were injected intraperitoneally with *E. coli* LPS serotype 0127:B8 at a concentration of 6.7 mg/kg body weight. This amount of LPS was selected as two-thirds of the lethal dose of LPS, which was pre-titrated in wild-type mice. For lethal endotoxicity determinations, age- and weight-matched female mice were injected with LPS and monitored at 8 h intervals. For the *in vivo* cytokine production, blood was obtained from the post-orbital vein complex at 1, 2, and 5 h after LPS challenge. Mice were euthanized immediately after the last bleed. Cytokine levels in serum were determined by ELISA.
Mammalian Two-hybrid Technique—The Check-Home
Mammalian Two-Hybrid System (Promega) was employed. The intact murine NF-κB1(p50) gene (GenBank™
NM_008689) (19, 23) was amplified, sequenced, and cloned in-frame into the pBIND vector, which contains the yeast
GAL4 DNA-binding domain. The murine CREB-binding
protein (CBP) (Gene bank NM_001025432) full-length
(167–2441) (38) were cloned in-frame into the pACT vector,
which contains the herpes simplex virus VP16 activation
domain and a nuclear localization sequence. A luciferase
reporter driven by five GAL4 binding sites (pG5-luc) was
cointroduced into RAW 264.7 cells, and luciferase activity
was measured.

Chromatin Immunoprecipitation Assay—ChIP assays
were performed following the instructions of the ChIP assay kit
(Upstate, Lake Placid, NY). The antibodies used were: α-p50
(SC-7178X), α-p65 (SC-109X), α-c-Rel (SC-71X, Santa Cruz
Biotechnology), and α-CBP (SC-369), which are all demonstrated
to be ChIP-grade antibodies (39–42). The presence of
associated transcription factors with their respective promotors
were measured by real-time PCR with the ABI Prism 7700
Sequence Detection System using SYBR Green PCR reagents
(Applied Biosystems). The primers used to amplify the IL-10
promoter were (−97/+35): sense, 5′-TAGGAGGAGGA-
GGAGCC-3′; antisense, 5′-TTGGCTTGTGGATGAGCAAG-
AGTA-3′. The upstream non-related IL-10 promoter region
(−1563/−1427) primers were: sense, 5′-CAGTCAAGAGGAGGCG-
AGTGA-3′; antisense, 5′-TTCTAACAGCAAGCAACACAC-
AGTA-3′. The primers for the −4652/−4322 region of the IL-
10 promoter are as reported (43). The primers for the murine
IL-12p35 promoter were 5′-CGACGCACTGGTCCTTTAGAT
and 3′-ACTGAGGAGGCTGCTGAT to amplify the region of
−167/−12 relative to the translation start codon
(GenBank™NM_058241), which has been demonstrated to contain
an NF-κB binding site (44). The thermocycler settings were: 1
cycle at 50 °C for 2 min; 1 cycle at 95 °C for 10 min; and 45 cycles
at 95 °C, for 15 s, 58 °C for 45 s, and 72 °C for 35 s. The PCR
products were checked on 1.5% agarose gel to confirm the spec-
ificity of amplification. The signal was normalized with the
signal from a B-actin reporter construct. The signal was
normalized with the signal from a B-actin reporter construct.

Electrophoretic Mobility Shift Assay—EMSA and supershift
assays were performed as previously described (17). The antibod-
ies used were: α-p50 (SC-114X), α-p65 (100–4165) (Rock-
land Immunochemicals, Gilbertsville, PA), α-c-Rel (SC-70X),
and normal rabbit IgG (SC-207, Santa Cruz Biotechnology).
The IL-12(p35) oligomer used for EMSA was TCTGGGAGA-
AGTCTTGCCGG, which contains the −110/−100 NF-κB site
as previously reported (44).

Reverse Transcription-PCR—Reverse transcription-PCR
was performed as described (13) using real-time PCR. The follow-
ing PCR primers were used. mL-10: sense, 5′-AAGG-
ACCAGCTGAGCAACAT-3′ and antisense, 5′-TCTCACC-
CAGGGAATTCAAA-3′; TNF-α: sense, 5′-CATCAGTTCT-
TATGGCCCAAGC-3′ and antisense, 5′-TGGGCTAC-
AGGCTTTGCACA-3′; mouse glyceraldehyde-3-phosphate
dehydrogenase: sense, 5′-TGTTCCTACCCCCCAATGTGT-3′
and antisense, 5′-GGTCCTCAGTGTTAGCCCAAAG-3′.

Statistical Analysis—Student t tests were performed where
indicated. For the survival data in Fig. 8D, a survival curves
square test was performed by GraphPad Prism 4 software
(GraphPad Software, Inc.). In all cases, differences of p < 0.05
are considered to be statistically significant.

RESULTS

The Contribution of NF-κB to IL-10 Transcription—We
undertook a series of studies to determine whether NF-κB was
involved in IL-10 transcription. Initially a murine IL-10 pro-
moter-driven luciferase construct (−1538/+64) was trans-
ferred into the macrophage-like cell line, RAW264.7, as previ-
ously described (14). This reporter construct responded to LPS
stimulation, resulting in an increase in luciferase activity (Fig.
1A). This reporter construct was also co-transfected along with
a p105-based NF-κB super suppressor (p105sr), which contains
a deletion in the cleavage domain, preventing signal-induced
degradation (34). Consequently, p105sr efficiently inhibits all
NF-κB activities, including p50 homodimers (34). The p105sr
effectively inhibited LPS-induced IL-10 promoter activities,
reducing luciferase activity to levels that were not different
from unstimulated (medium) cells (Fig. 1A). We then truncated
the IL-10 promoter to −158/+64 and performed similar stud-
ies. Again this reporter responded to LPS stimulation, and this
response was completely blocked by p105sr (Fig. 1A).

Similar studies were also performed with a dominant positive
IκB-α mutant plasmid (IκB-α mut), which encodes a protein
involving a deletion of N-terminal amino acids 1–36, lacking the
 phosphorylation sites necessary for ubiquitination and degra-
dation. Thus, it is a pan-NF-κB signaling blocker (45). The
IκB-α mut had similar inhibitory activity to p105sr, reducing
luciferase levels to that observed in unstimulated cells (data not
shown).

Because the proximal reporter preserved most of the LPS-
induced luciferase activity, we focused on the proximal region
of the IL-10 promoter to address NF-κB responsiveness. To
determine which of the NF-κB/Rel family members were
involved in IL-10 induction, expression plasmids encoding the
five NF-κB/Rel family members, including RelA (p65), NF-κB1
(p50), NF-κB2 (p52), c-Rel, and RelB (19) were co-transfected
into cells with pIL-10(−158/+64)-luc. Only the overexpression
of p50 induced substantial levels of IL-10-driven luciferase
activity (Fig. 1B). None of the other Rel family members
resulted in increased luciferase activity. The p50 homodimer-
induced IL-10 promoter activity responded not only to LPS
stimulation (Fig. 1B), but also to several other TLR agonists,
including Pam2 (2) and Pam3(3)CSK4 (supplemental Fig. S1).

To determine whether p50 worked alone or paired with one
of the other NF-κB/Rel family members, a co-transfection
approach was taken, using a combination of Rel family
members. Similar to Fig. 1B, the expression of p50 alone resulted in a
significant induction luciferase activity in response to LPS (Fig.
1C). Co-transfecting either p65 or c-Rel together with p50,
however, resulted in a substantial decrease in luciferase activity
relative to that of p50 alone (Fig. 1C). These studies suggest that
NF-κB participates in the IL-10 transcriptional regulation and
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Figure 1. NF-κB (p50) up-regulates IL-10 promoter activity. A, 1 × 10⁷ log phase RAW264.7 cells were co-transfected with 3 μg of the luciferase reporter constructs driven by the IL-10 promoter (pIL-10(−1538/+64)-luc and pIL-10(−158/+64)-luc) and 3 μg of a p105-based super repressor (p105sr) or its control vector (pEVRF). 24 h later, cells were stimulated with 10 ng/ml LPS (solid bars), and luciferase activity was measured in cell lysates 16 h later. Significant differences are designated: *, p < 0.01 compared with vector controls. B, 3 μg of pIL-10(−158/+64)-luc along with 3 μg of each of the five NF-κB/Rel members (NF-κB1 (p50), RelA (p65), c-Rel, NF-κB2 (p52), and RelB) expression constructs were transfected into RAW cells, and the luciferase activity was measured. C, 3 μg of pIL-10(−158/+64)-luc along with individual NF-κB/Rel members, or different pairs of NF-κB/Rel members together. Luciferase activity was measured in cell lysates 16 h after stimulation with LPS. **, p < 0.01 compared with p50 alone.

Figure 2. A characterization of the −59/−39 NF-κB1(p50) binding site. A, 32P-end-labeled double-stranded oligomers corresponding to −59/−39 sequence (above the figure) were incubated with 0.4 gel shift units of rhNF-κB1 (p50) protein, in the presence of increasing amounts (from 2.5- to 25-fold molar ratio) of non-labeled double-stranded oligomers (−59/−39), an NF-κB consensus (Cons), or a mutant NF-κB oligomer (Mut), whose sequences are shown above the figure. B, the same 32P-end-labeled double-stranded oligomers corresponding to the mIL-10 promoter (−59/−39) were incubated with 5 μg of nuclear extract isolated from BMMφs treated with 10 ng/ml LPS for 1 h. Supershift analysis was performed with antibodies to p50, p65, and c-Rel, or with normal rabbit IgG as a control. Free probe alone is shown in lane 1. The asterisk designates supershift.

that p50 is the only Rel family member to drive IL-10 transcription. The other NF-κB/Rel family members not only fail to contribute to IL-10 transcription, they can actually inhibit it.

The Binding of p50 to the IL-10 Promoter—To dissect the NF-κB binding site(s) in the murine IL-10 promoter, the MatInspector program (46) was used to analyze a portion of the proximal IL-10 promoter. We combined this with an EMSA approach using recombinant p50. The −59/−39 site of the IL-10 promoter bound recombinant p50, and this binding was competed for by increasing molar ratios of a cold oligomer corresponding to this site (Fig. 2A). The binding of p50 to this site was also competed for by a cold consensus NF-κB element (Fig. 2A). However, a mutant form of the consensus oligomer, containing a single base mutation (G → C) in the NF-κB binding site failed to compete for binding (Fig. 2A). A similar competitive EMSA was done with another single base substitution (−48C → G). This mutant also failed to compete for binding (supplemental Fig. S2A).

EMSA and supershift analysis were also performed using nuclear extracts from LPS-stimulated primary murine macrophages (Fig. 2B). Nuclear extracts from stimulated cells bound to the −59/−39 element (Fig. 2B). The addition of antibody to p50 resulted in a supershift (Fig. 2B), however antibodies to p65 and c-Rel failed to supershift (Fig. 2B). In contrast to IL-10, these same antibodies to p65 and c-Rel supershifted NF-κB from the IL-12 promoter (supplemental Fig. S2B). The binding of nuclear extracts to the IL-10 promoter was competed for by a cold oligomer corresponding to the −59/−39 site and by a consensus NF-κB oligomer (supplemental Fig. S2C). However, binding was not competed for by an NF-κB oligomer containing a single base substitution (supplemental Fig. S2C), indicating that the binding was specific to this sequence. Similar EMSA and supershift results were observed with nuclear extracts from stimulated RAW264.7 cells (data not shown). These results indicate that p50 binds to the −59/−39 site of the IL-10 promoter.

To demonstrate that the p50-binding site was biologically relevant, a plasmid containing a minimal IL-10 promoter (−82/
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FIGURE 3. Functional analysis of the NF-κB binding sites. RAW264.7 cells were transfected with 2.5 μg of a p50 expression plasmid, along with 3 μg of pIL-10(-82/+64)-luc wild-type (open bar), or pIL-10(-82/+64)-luc M1(-48C → G) (gray bar), or pIL-10(-82/+64)-luc M2(-22G → C) (black bar), or pIL-10(-82/+64)-luc M1/M2 (striped bar). The sequences of the four constructs are shown below the figure. 24 h later the cells were stimulated with 10 ng/ml LPS for 16 h, and the cell lysates were prepared for luciferase assays. The activities for each of the reporter constructs are plotted relative to the wild-type promoter-driven activity, which was set at 100%. This figure is representative of three.

FIGURE 4. NF-κBp50 binds to murine IL-10 proximal promoter in situ. A, BMMs derived from BALB/c mice were stimulated with 10 ng/ml of LPS for the indicated times, and processed for ChIP analysis. The kinetic fold change in signal is reported relative to time 0. Amplification of the −97/+35 region of murine IL-10 promoter is shown following immunoprecipitation with anti-p50 (solid line and black circles), p65 (dotted line and inverted triangles), or c-Rel (dashed line and squares). B, as a control, the −167/−12 region of murine IL-12p35 promoter was amplified. TFs, transcription factors.

Distinct NF-κB Members Differentially Regulate Pro- and Anti-inflammatory Cytokine Production—It has been well known that NF-κB signaling participates in inflammatory cytokine production (19). As controls for the IL-10 promoter, we examined the promoters of some of these inflammatory cytokines. We examined p50, p65, and c-Rel, in the regulation of TNF and IL-12, and compared this with IL-10. For the IL-12 p40 (Fig. 5B) and p35 (Fig. 5C) promoters, and for the TNF promoter (Fig. 5D), p50 played little or no role in driving luciferase activity. For all three of these inflammatory cytokine reporters, c-Rel and p65 play dominant roles in driving transcription. This is the opposite of what we observed for IL-10 (Fig. 5A), where p65 and C-Rel play little to no role in activating the IL-10 promoter. Thus, p50 plays a dominant role in promoting IL-10 expression; however, it plays a minimal role in inducing the transcription of the two inflammatory cytokines that we examined.

The Identification of p50-interacting Proteins—The identification of p50 as the single NF-κB family member driving IL-10 expression was initially unexpected because p50 homodimers have traditionally been thought of as transcription repressors, due to the lack of a transcriptional activation domain (19). Thus, to induce transcriptional activation, p50 is presumed to pair with other activating factors. Therefore, we examined several potential p50-interacting factors, including Bcl-3 (25, 47), C/EBPa, C/EBPβ (48, 49), and p300 (26, 50). Our approach to examine these factors was to employ a co-transfection approach to determine whether the overexpression of any of these factors could increase p50-mediated IL-10 transcription. The co-transfection of p50 along with Bcl-3, C/EBPa, C/EBPβ, or p300 resulted in no significant increases in IL-10 promoter-driven luciferase activity (supplemental Fig. S4). Because the...
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FIGURE 5. Individual NF-κB/Rel family members differentially regulate cytokine production. A, 1 × 10⁷ log phase RAW264.7 cells were co-transfected with 3 μg of pIL-10(−158/+64)-luc reporter along with 3 μg of each of p50, p65, or c-Rel expression constructs, or their control vector. 24 h after electroporation, the cells were treated with 10 ng/ml LPS for 16 h, and cell lysates were prepared for luciferase assays. B-D, similar assays were performed with different cytokine promoter-driven luciferase reporters: IL-12p40 (5 μg) (B), IL-12p35 (3.5 μg) (C), or TNF-α (2.5 μg) (D). For TNF-α promoter analysis cells were stimulated with 10 ng/ml LPS for 6 h. For the IL-12p40 and p35 promoters, the cells were primed with 100 units/ml interferon-γ for 12 h and then treated with 10 ng/ml LPS for 6 h. This figure is representative of three.

co-transfection of p50 and Bcl-3 increased IL-10 promoter activity slightly at the very early times (supplemental Fig. S4A), macrophages from Bcl-3 knock-out mice were examined for IL-10 production. These macrophages exhibited no defect in LPS-induced IL-10 production (supplemental Fig. S4B). Thus, none of these factors are likely to be candidates for p50-interacting partners inducing IL-10 transcription.

In contrast to these factors, the CREB-binding protein, CBP, provided the most compelling evidence for a factor that could associate with p50 and activate IL-10 transcription. The co-transfection of CBP along with p50 resulted in a significant increase in IL-10-driven luciferase activity (Fig. 6A), suggesting that CBP may partner with p50 to induce IL-10 transcription. This analysis was repeated comparing this CBP-dependent induction of the wild-type promoter to that of a promoter with the single base mutation (M1), described in Figs. 3 and S2. This mutated promoter failed to respond to either p50 or to p50+CBP induced activation (Fig. 6B).

A ChIP analysis was performed on stimulated cells, and this assay verified that CBP binds to the proximal IL-10 promoter in situ in LPS-stimulated primary macrophages (Fig. 6C), although the kinetics of CBP association appeared to be slightly different from that of p50 (Fig. 6C).

To determine whether CBP and p50 could associate in cells, CBP was immunoprecipitated from stimulated macrophages and p50 was found to co-precipitate with it by Western blotting (Fig. 6D). Neither p65 nor c-Rel could be detected in association with CBP by this approach (Fig. 6D). To more closely examine the interaction of CBP with p50, a mammalian two-hybrid approach was taken, using full-length and truncated forms of CBP. The CBP constructs were cloned into a vector containing the VP16 activation domain and co-transfected into RAW264.7 cells along with p50 that was cloned into a vector containing the Gal4 binding domain. In this system, if p50 and CBP interact it will result in Gal4 multimer-driven luciferase activity. The construct encoding intact CBP provided a significant increase in Gal4-dependent luciferase activity (Fig. 6E), of which the C-terminal portion of CBP had the highest activity. This induction by the C-terminal domain of CBP exceeded even that of intact full-length CBP (p < 0.001) (Fig. 6E).

To further verify that p50 could interact with CBP, we took advantage of previous observations that the adenovirus E1a gene could associate with CBP and competitively inhibit transcription factor binding to CBP (32). RAW264.7 cells were co-transfected with E1a and the IL-10-luciferase construct and stimulated with LPS. The overexpression of E1α in macrophages completely blocked IL-10 luciferase activity driven by p50 alone or by p50 plus CBP (Fig. 6F), further demonstrating that CBP is required for the promotion of p50-driven transcription of IL-10.

Cytokine Production in NF-κB1 (p50) Knock-out Mice—To verify a role for p50 in the induction of IL-10 cytokine production, BMMΦs from mice lacking p50 were stimulated for various times with LPS, and then cytokine production was measured by ELISA. Macrophages from mice lacking p50 produced reduced amounts of IL-10 protein (Fig. 7A) and mRNA (Fig. 7B) relative to wild-type mice. In contrast to IL-10, these macrophages produced slightly increased amounts of IL-12, and dramatic increases in TNF (Fig. 7C). To verify that the decreased IL-10 production in the macrophages lacking p50 was in fact due to the lack of p50, BMMΦs from NF-κB1−/− mice were reconstituted with a murine p50 expression construct, using nucleofection. The efficiency of transfection was ~90% (supplemental Fig. S5). BMMΦs from NF-κB1−/− mice produced decreased IL-10 production relative to wild-type mice (Fig. 7D). The transfection of these cells with an expression plasmid encoding p50, however, resulted in a recovery of IL-10 production in response to LPS stimulation that was not significantly different from wild-type macrophages (p > 0.05) (Fig. 7D).
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NF-κB1 produced lower amounts of IL-10 relative to normal mice (Fig. 8A), but higher levels of IL-12 and TNF (Fig. 8B). The decreased production of IL-10 and the increased production of IL-12 and TNF predicted that mice lacking p50 would be more susceptible to lethal endotoxemia. To test this, in vivo lethality experiments were undertaken to compare the sensitivity of NF-κB1−/− mice to endotoxin with wild-type mice. A sublethal dose of 6.7 mg/kg LPS was selected by titration in wild-type mice. This amount was then administered to wild-type and p50−/− mice. 80% of the mice lacking p50 succumbed to lethal endotoxemia within 2 days of LPS administration, whereas only 10% of wild-type mice died (Fig. 8C). Thus, the lack of NF-κBp50 predisposes mice to lethal endotoxemia.

**DISCUSSION**

Although the role of the NF-κB family in promoting the production of inflammatory cytokines has been well described (19), their role in promoting IL-10 transcription is not as clear (14, 51, 52). In the present study we examined a role for NF-κB in IL-10 gene regulation. We present four major pieces of evidence to support the conclusion that NF-κB is involved in IL-10 induction. First, blocking NF-κB signaling by a p105-based NF-κB super repressor completely blocked LPS-induced IL-10 promoter activity (Fig. 1A). Second, by overexpressing NF-κB1 (p50) we can significantly up-regulate IL-10 promoter activity. None of the other four members (p65, p52, c-Rel, or RelB) had this activity (Fig. 1B). Third, BMMs derived from BALB/c mice were stimulated with 10 ng/ml LPS for 16 h, and the luciferase was determined. Significant differences are designated: *, p < 0.01 compared with LPS-stimulated vector; **, p < 0.05, compared with LPS-stimulated p50 using unpaired two-tailed Student’s t test. BMMs derived from BALB/c mice were stimulated with 10 ng/ml LPS for the indicated times, and processed for ChIP analysis. Amplification of the −97/+35 region of murine IL-10 promoter is expressed as fold change relative to time 0 following immunoprecipitation with anti-CBP (dotted line and inverted triangles), p50 (solid line and circles), or rabbit IgG (dashed line and squares). TFS, transcription factors. D, nuclear extracts were prepared from RAW 264.7 cells treated with 10 ng/ml LPS for 2 h. 800 μg of nuclear extracts was precipitated with antibody to CBP, and then detected with anti-p50, -p65, or -c-Rel by Western blotting. E, RAW 264.7 cells were co-transfected with a 3 μg of pG5-luciferase construct, together with 3 μg of the pBIND plasmid into which p50 was cloned in-frame (pBIND/p50), along with 3 μg of pACT/CBP encoding either full-length (amino acids 1–2441) or the C-terminal domain (amino acids 1758–2441) of CBP. 24 h after transfection the cells were treated with 10 ng/ml LPS for 16 h, and the luciferase was determined. The fold-induction of luciferase activity is plotted relative to the activity induced by the two empty vectors, pBIND and pACT, which was set to one. *, p < 0.01 compared with control (pBIND) group; **, p < 0.001 compared with pACT 1–2441. F, RAW264.7 cells were co-transfected as in A, in the presence or absence of an additional E1a expression plasmid (3 μg) (open bars, not visible). 24 h later, cells were treated with 10 ng/ml LPS for 16 h, and the absolute luciferase activities are shown.

The Role of p50 in Mediating Host Responses to Endotoxin—

To examine the in vivo response to endotoxin, mice were injected with LPS. Plasma cytokine levels were analyzed by ELISA at various times thereafter. Knock-out mice lacking KO mice had lower levels of IL-10 and were hypersusceptible to lethal endotoxemia (Fig. 8).

It is important to acknowledge that NF-κB1 KO mice can still produce low levels of IL-10, suggesting that there are p50-inallel-
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FIGURE 7. BMMøs from NF-κB1 knock-out mice exhibit altered cytokine responses to LPS. A and B, 6 × 10⁷/ml BMMøs from NF-κB1 KO (dashed lines and triangles) and control wild-type mice (solid lines and squares) were treated with 10 ng/ml LPS for the indicated times. Supernatants were analyzed to determine the levels of IL-10 protein by ELISA (A), or mRNA by reverse transcription-PCR (B). C, the production of IL-12 (triangles) and TNF-α (inverted triangles) by BMMøs from NF-κB1 KO (dashed lines) and control wild-type (solid lines) mice following stimulation with 10 ng/ml LPS for the indicated times. D, BMMøs from NF-κB1 knock-out and control mice were reconstituted with an NF-κBp50 expression vector (solid bars) or its control plasmid (open bars) by nucleofection. 24 h later the transfected cells were treated with 10 ng/ml LPS for 16 h. The levels of IL-10 were determined by ELISA. *, a significant increase (p < 0.01) in IL-10 production relative to vector controls; **, a significant decrease (p < 0.01), relative to wild-type.

A dependent mechanism(s) in place, which can induce IL-10 transcription in the absence of NF-κBp50. Constitutively expressed transcription factors, such as Sp-1 and Sp-3, would be candidates for NF-κB-independent mechanisms, both of which have been shown to participate in IL-10 regulation (14, 53). The participation of many different transcription factors and the multiple levels of gene regulation would be in agreement with the idea that the regulation of IL-10 production is complex and tightly regulated (13).

Another observation made by this work is that NF-κB1 (p50) differently regulates the production of inflammatory and anti-inflammatory cytokine production. Because there is clear evidence that NF-κB signaling is involved in the induction of IL-12 and TNF-α (19), we used these two inflammatory cytokine genes as controls for p50-mediated IL-10 transcriptional activation. We show several lines of evidence that are consistent with the idea that distinct NF-κB/Rel family members differentially regulate pro- and anti-inflammatory cytokine production. First, using promoter-driven reporter studies we demonstrated that p50 plays little role in the induction of TNF or IL-12 (Fig. 5). Rather, these genes were regulated primarily by p65 and c-Rel, respectively, a finding that is consistent with previous observations of others (31). Conversely, we show that p65 and c-Rel play no role in driving IL-10 expression (Fig. 1). Second, cytokine production in NF-κB1 KO mice shows a reciprocal regulation between pro- and anti-inflammatory cytokine production. Macrophages isolated from mice lacking p50 produced higher levels of TNF and IL-12 but reduced levels of IL-10 in response to LPS (Fig. 7). As a result, these mice are more susceptible to lethal endotoxemia. Although previous investigations have suggested that p50 can pair with c-Rel or p65 to induce IL-12 and TNF (19), the increased production of these two cytokines in the p50 KO mice, along with the inability of p50 overexpression to drive TNF or IL-12 promoter activity (Fig. 5), suggests that p50 plays only a minor role, if any, in activating TNF or IL-12. In fact, our observations are more consistent with the idea that p50 somehow inhibits the production of these inflammatory cytokines.

It is well appreciated that p50 homodimers can act as suppressors of inflammatory cytokine genes (19). We suggest an additional mechanism of control whereby the relative amounts of each of the NF-κB/Rel family members may affect the character of the innate immune response that develops. We propose that the ratio of p50 relative to the other Rel family members in the nucleus is likely to be a determining factor for gene expression. We predict that high levels of p65 and c-Rel can compete for p50 binding to itself and thereby diminish p50 homodimer formation, and subsequently IL-10 production. This hypothesis is consistent with the observations of Mori and Prager (54) who reported that IL-10 expression is related to high NF-κBp50 expression in a T-cell line. It may also be consistent with the observations of Nemeth et al. (52) who showed that inhibiting the “activating” NF-κB members (55) with pyrrolidine dithiocarbamate could actually up-regulate IL-10 production. All of these assumptions will need to be further examined.

The third observation to emerge from these studies is that NF-κB homodimers interact with the transcriptional co-activator, CBP, in activating IL-10 transcription. We examined several potential p50-interacting proteins and excluded them as inducers of IL-10 transcription. Because Bcl-3, C/EBP, and p300 have been shown to associate with p50 to transactivate other genes (25, 26, 48), we tested all three of these genes using a reporter approach. The co-transfection of p50 with any of these potential co-activators failed to result in significant increases in IL-10 promoter activity. Furthermore, Bcl-3 KO BMMøs were found to have no defect in IL-10 production, suggesting that Bcl-3 is not involved in IL-10 production, as recently suggested (56). Therefore, we examined the interaction of p50 with CBP. We demonstrated that these two molecules can increase the IL-10 promoter activity, and that they can physically interact with each other (Fig. 6). We were unable to...
detect the interaction of p65 or c-Rel with CBP by co-immunoprecipitation. To address which of the one or more domains of CBP interact with p50, a mammalian two-hybridization approach was taken. The results show that the C-terminal portion of CBP has the strongest activity in this assay (Fig. 6D). Previous observations of others have shown that RelA (p65) can interact with CBP at the N terminus (38) and that p65 can compete with CREB for CBP binding (57). Although in our system we could not detect an association of p65 with CBP, further studies to examine the interaction of Rel family members with CBP under various experimental conditions are clearly warranted.

The fourth observation to emerge from this work was the identification of the NF-κB binding site in the proximal murine IL-10 promoter. Competitive EMSAs, supershift experiments, and functional studies revealed that p50 binds to the −55/−46 site and induces transcription. A ChIP assay verified that p50 preferentially binds in situ to this region of IL-10 promoter (Fig. 4). Taken together, these studies identify a novel p50-responsive cis-element in IL-10 promoter. Studies are underway to determine whether other members of the IL-10 gene family (3) are similarly regulated by p50 homodimers.

In conclusion, our study demonstrates that NF-κB participates in IL-10 transcription. We show that NF-κB (p50) homodimers bind to the proximal IL-10 promoter and form a complex with co-activators, such as CBP to activate IL-10 transcription. We also show that p50 homodimers differentially regulate IL-10 versus TNF and IL-12. We suggest that p50 homodimers can have opposing effects with regard to cytokine production. On one hand, p50 homodimers can be the brake, which prevents the production of pro-inflammatory cytokines by forming repressive homodimers, whereas on the other they can be the engine that drives anti-inflammatory cytokine production by binding to the IL-10 promoter and activating transcription. The further characterization of the distinct NF-κB members in the regulation of pro- and anti-inflammatory cytokines and the understanding of other p50-interacting partners would take us a step closer to being able to manipulate p50 levels in macrophages to either enhance immunity or prevent autoimmunity.

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