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# Redundancy of C/EBP $\alpha$ , - $\beta$ , and - $\delta$ in Supporting the Lipopolysaccharide-Induced Transcription of IL-6 and Monocyte Chemoattractant Protein-1<sup>1</sup>

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C/EBP $\alpha$ , - $\beta$ , and - $\delta$  are members of the CCAAT/enhancer binding protein family of transcriptional regulators. All three of these factors are expressed by bone marrow-derived macrophages, with the DNA binding activity of C/EBP $\beta$  and - $\delta$  increased by treatment with LPS while that of C/EBP $\alpha$  is decreased. We have ectopically expressed each C/EBP protein in P388 lymphoblasts. The expression of any of these transcription factors is sufficient to confer the LPS-inducible expression of IL-6 and monocyte chemoattractant protein-1 to lymphoblasts, which normally lack C/EBP factors and do not display LPS induction of proinflammatory cytokines. Thus, the activities of C/EBP $\alpha$ , - $\beta$ , and - $\delta$  are redundant in regard to the expression of IL-6 and monocyte chemoattractant protein-1. Since C/EBP $\beta$ -deficient mice have been reported to be largely normal in their expression of proinflammatory cytokines, it is likely that the lack of C/EBP $\beta$  is compensated for by the induction of C/EBP $\delta$  upon LPS treatment. *The Journal of Immunology*, 1998, 160: 2334–2342.

C/EBP-related proteins comprise a family of basic region-leucine zipper transcription factors (reviewed in Ref. 1). These proteins dimerize through a leucine zipper and bind to a consensus DNA motif through an adjacent basic region. C/EBP-related transcription factors have been implicated in the regulation of a number of proinflammatory cytokines as well as other gene products associated with the activation of macrophages by microbial products and cytokines. For example, the promoter regions of the genes for IL-6, IL-1 $\alpha$ , IL-1 $\beta$ , IL-8, TNF- $\alpha$ , G-CSF,<sup>3</sup> nitric oxide synthase, and lysozyme (2–7) contain C/EBP binding motifs. Furthermore, both C/EBP $\beta$  and C/EBP $\delta$  can *trans*-activate a reporter gene regulated by the IL-6 promoter in transient expression assays (2, 8). We have previously shown that the stable expression of C/EBP $\beta$  in a murine B lymphoblast cell line can confer the ability to induce IL-6 and MCP-1 expression with LPS (9).

Two groups of investigators have recently generated mice deficient for C/EBP $\beta$  expression (10, 11). Tanaka et al. (11) found that LPS stimulation of peritoneal macrophages from such animals led to a normal induction of a number of proinflammatory cytokines, including IL-6. Basal levels of IL-6 mRNA were, in fact,

elevated. These animals' macrophages, however, failed to express G-CSF mRNA in response to LPS stimulation. Screpanti et al. (10) found C/EBP $\beta$ -deficient mice to have elevated levels of IL-6 expression, but did not otherwise report the ability of macrophages from those mice to produce proinflammatory cytokines. Consistent with the findings of Tanaka et al. (11), ablation of C/EBP $\beta$  expression in human fibroblasts with either antisense- or ribozyme-mediated elimination of C/EBP $\beta$  mRNA blocked TNF- $\alpha$  induction of G-CSF, but not IL-6 expression (12).

The above results indicate that C/EBP $\beta$  is not necessary for the induction of IL-6 in the inflammatory response. However, the requirement of a C/EBP activity for LPS induction of IL-6 is very likely, since we have previously demonstrated a critical role for C/EBP $\beta$  in this process (9). Several monocyte and macrophage cell lines have been reported to express both C/EBP $\beta$  and C/EBP $\delta$  (9, 8), and immature myelomonocytic cell lines have also been reported to express C/EBP $\alpha$  (13). It is thus reasonable to propose that the expression of IL-6 and other proinflammatory cytokines by the macrophages of C/EBP $\beta$ -deficient mice is supported by C/EBP $\delta$  or, perhaps, C/EBP $\alpha$ . C/EBP $\alpha$ , C/EBP $\beta$ , and C/EBP $\delta$  have all been reported to be functional in a heterologous transgenic rescue assay for a *Drosophila* C/EBP mutant, slow border cells (14), but the functional redundancy of C/EBPs in cytokine expression in mammalian cells has not been demonstrated. In this report we have directly compared the capacities of C/EBP $\alpha$ , C/EBP $\beta$ , and C/EBP $\delta$  to confer LPS-induced cytokine expression to a lymphoblastic cell line normally lacking this capability. Using stable transfection and endogenous cytokine genes containing a full complement of regulatory sequences, we show that any one of these C/EBPs can confer LPS-inducible expression of the genes encoding IL-6 and MCP-1. These results demonstrate the redundancy of C/EBP $\alpha$ , C/EBP $\beta$ , and C/EBP $\delta$  in supporting the LPS induction of IL-6 and MCP-1.

## Materials and Methods

### Cells and cell culture

Bone marrow-derived macrophages were obtained from C57 Black/6 mice. Bone marrow was explanted from femurs into DMEM supplemented with

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<sup>3</sup> Abbreviations used in this paper: G-CSF, granulocyte CSF; MCP-1, monocyte chemoattractant protein-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay; NF- $\kappa$ B, nuclear factor- $\kappa$ B; MIP-1 $\alpha$ , macrophage inflammatory protein-1 $\alpha$ .

10% FCS, 10% heat-inactivated horse serum, and 20% L cell-conditioned medium at a density of  $10^7$  cells/ml in 25 ml on 150-mm tissue culture plates. After 48 h, the nonadherent cells were removed and replated at a density of  $3 \times 10^5$  cells/ml in 10 ml on 100-mm tissue culture plates. Culture continued for 7 days, with a change of medium every 3 days.

P388 cells are murine B lymphoblasts (15) (American Type Culture Collection, Rockville, MD; CCL46). P388-C $\beta$  cells are P388-C2 cells previously described by Bretz et al. (9). Cells were cultured in RPMI 1640 medium supplemented with 5% FCS and 50  $\mu$ M 2-ME. Inductions were conducted with LPS derived from *Escherichia coli* serotype 055:B5 (Sigma Chemical Co., St. Louis, MO) added to 10  $\mu$ g/ml.

### Transfections

Transfections of G418-resistant vectors were conducted with  $10^6$  cells, 5  $\mu$ g of DNA, and 40  $\mu$ g of lipofectin (Life Technologies, Grand Island, NY) in 3 ml of Opti-MEM I medium (Life Technologies). Cells were incubated in the transfection mixture for 16 h followed by the addition of RPMI 1640 supplemented with 20% FCS. After 72 h, the medium was replaced with the standard growth medium supplemented with G418 (Life Technologies) at 0.67 mg/ml. Transfections of puromycin-resistant vectors were conducted similarly with a selective concentration of puromycin (Boehringer Mannheim, Indianapolis, IN) at 7  $\mu$ g/ml.

### Expression vectors

pSV(X)Neo is pZIP-NEO SV(X)1 (16) and uses the promoter of Moloney murine leukemia virus. pSV(X)C/EBP $\alpha$  was constructed by insertion of the *Bam*HI/*Kpn*I fragment encoding rat C/EBP $\alpha$  from pMEXC/EBP (17) into the *Bam*HI site of pSV(X)Neo with *Bam*HI linkers. pSV(X)C/EBP $\beta$  was constructed by insertion of the *Bam*HI fragment encoding rat C/EBP $\beta$  from pMEXCRP2 (17) into the *Bam*HI site of pSV(X)Neo. To construct an expression vector for C/EBP $\delta$ , the sequences encoding murine C/EBP $\delta$  (17) were first inserted into the *Sph*I and *Hind*III sites of pMEX (17) by a three-part ligation; one inserted fragment extended from a PCR-introduced *Sph*I site 40 bp upstream of the C/EBP $\delta$  initiation codon to an *Apa*I site approximately 100 bp into the coding sequence, and the other fragment extended from the *Apa*I site to a PCR-introduced *Hind*III site just downstream of the termination codon. The *Sph*I/*Hind*III fragment was then inserted with *Bam*HI linkers into the *Bam*HI site of pSV(X)Neo to produce SV(X)C/EBP $\delta$ . The same *Bam*HI fragment was inserted into the *Bam*HI site of pBABE-Puro (18) to construct pBABE-C/EBP $\delta$ .

### Nucleic acid isolation and analysis

Total RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer's directions. RNAs were electrophoresed through 1% agarose/formaldehyde gels. Transfers to membranes were hybridized and washed to a stringency of  $0.1 \times$  SSPE at 65°C. Hybridization probes were prepared with a random priming kit (Life Technologies) with the incorporation of 5'-[ $\alpha$ - $^{32}$ P]dATP (3000 Ci/mmol; DuPont-New England Nuclear, Newton, CT). The IL-6 probe was a 0.65-kb murine cDNA (from N. Jenkins and N. Copeland, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD). The MCP-1 probe was a 0.58-kb murine cDNA (19). The GAPDH probe was a 1.3-kb rat cDNA (20).

### Western analysis

Nuclear extracts were prepared as described below. The extracts (20  $\mu$ g) were adjusted to  $1 \times$  Laemmli sample buffer (21) and processed on a 12% PAGE gel. The gel was transferred to a Protran membrane (Schleicher and Schuell, Keene, NH), and Ag-Ab complexes were visualized with the enhanced chemiluminescence kit (Amersham, Arlington Heights, IL).

### Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described by Lee et al. (22), except that the samples were not dialyzed into buffer D. Protein was incubated with a double-stranded oligonucleotide probe containing an optimal C/EBP binding site (5'-GATCTAGATATCCCTGATTGCGCAATAG-GCTCAAAGCTG-3' annealed with 5'-AATTCAGCTTTGAGCCTATTGCCCAATCAGGGA-TATCTAG-3') or to a probe homologous to the NF- $\kappa$ B binding site of the Ig  $\kappa$  light chain enhancer (5'-TCGACTCCCTGGG GACTTCCAGGCTCC-3' annealed with 5'-TCGAGGAGC-CTG GAAAGTCCCAGGGAG-3'). A probe containing a CTF/NF-1 consensus binding site (23) was used as a nonspecific competitor in some assays (5'-GATCCTTTGGCATGCGCAATA-TG-3' annealed with 5'-AATTCATATTGGCAGCATGCCAAAG-3'). Underlined sequences correspond to the binding motifs of the specified transcription factors. All binding reactions were performed at 23°C in a 25- $\mu$ l mixture containing 6  $\mu$ l

of nuclear extract (1 mg/ml in buffer C), 6% (v/v) glycerol, 4% (w/v) Ficoll, 10 mM HEPES (pH 7.9), 10 mM DTT, 0.25  $\mu$ g of BSA, 0.06% (w/v) bromophenol blue, 1  $\mu$ g of poly(dI-dC), and 1.25 ng of probe. Samples were electrophoresed through 5.5% polyacrylamide gels in  $1 \times$  Tris-Borate (pH 8.3) and 0.5 mM EDTA at 150 V. For supershifts, nuclear extracts were preincubated with antisera for 30 min at 4°C before the binding reaction.

### Antisera

Rabbit anti-C/EBP $\alpha$  was generated by immunization with a peptide corresponding to amino acids 253 to 268 of rat C/EBP $\alpha$  (23). Rabbit anti-C/EBP $\beta$  was generated by immunization with a peptide corresponding to amino acids 1 to 12 of C/EBP $\beta$  (17) or was purchased from Santa Cruz Biotechnology (Santa Cruz, CA; C/EBP $\beta$ ; C-19). Rabbit anti-C/EBP $\delta$  was obtained from M. Hannink (University of Missouri-Columbia) or was purchased from Santa Cruz Biotechnology (C/EBP $\delta$ ; C-22). Rabbit anti-C/EBP $\epsilon$  was purchased from Santa Cruz Biotechnology (CRP-1; C-22). Rabbit panCRP antiserum was generated by immunization with a peptide corresponding to a conserved motif within the basic region of C/EBP family members (24). Rabbit anti-p50 and anti-p65 were obtained from N. Rice (National Cancer Institute-Frederick Cancer Research and Development Center).

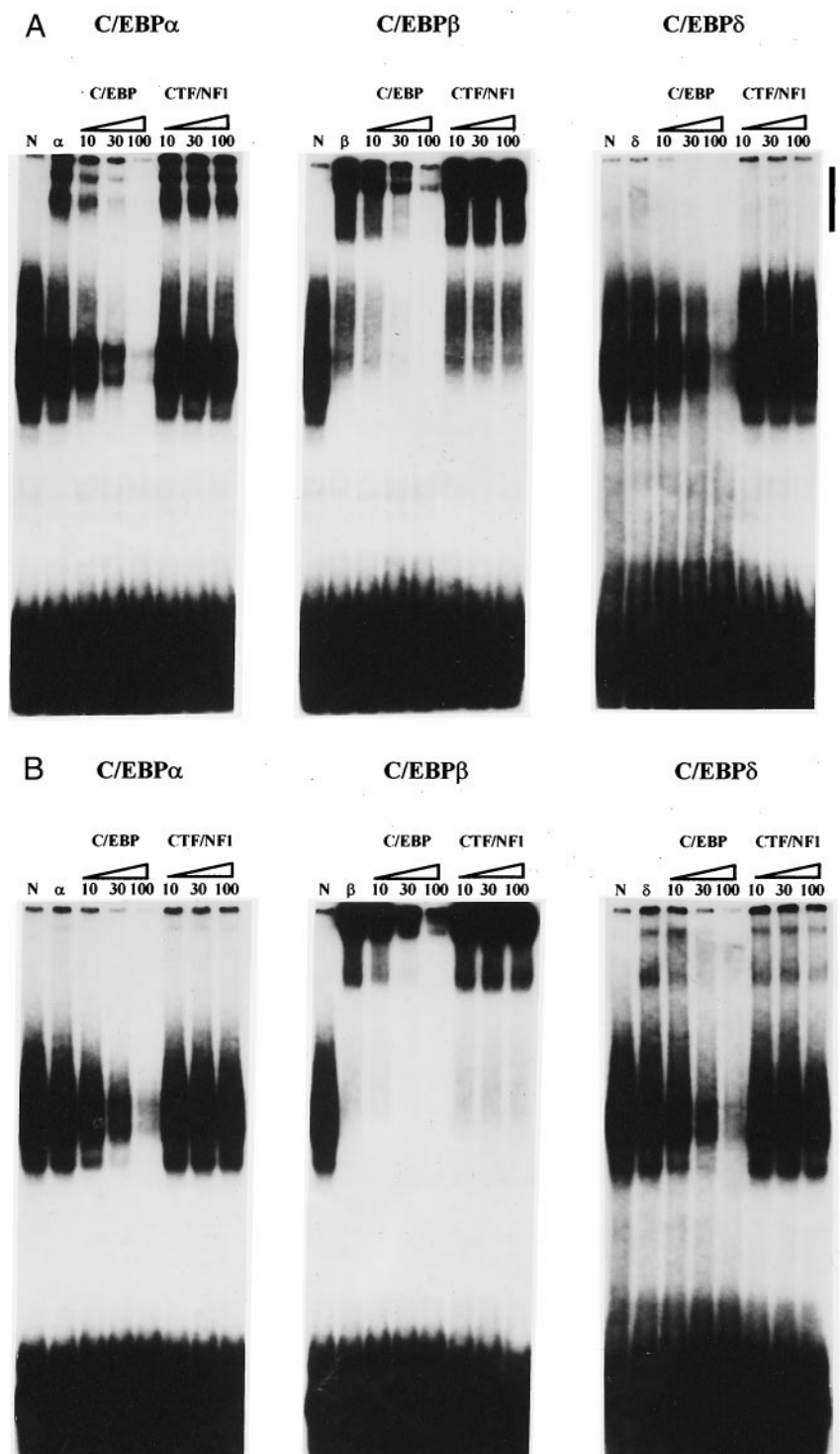
## Results

### C/EBP $\alpha$ , C/EBP $\beta$ , and C/EBP $\delta$ are all expressed in primary bone marrow-derived macrophages

To determine which C/EBPs are expressed in primary macrophages, EMSAs were performed on the nuclear extracts of bone marrow-derived macrophages. Supershifts with specific antisera revealed both C/EBP $\alpha$  and C/EBP $\beta$  DNA binding activities before LPS stimulation (Fig. 1A). C/EBP $\beta$  became the predominant binding species after treatment with LPS for 4 h; however, C/EBP $\alpha$  binding species were still present at a low level, and C/EBP $\delta$  binding species were induced (Fig. 1B). Thus, C/EBP $\alpha$ , C/EBP $\beta$ , and C/EBP $\delta$  are all potentially available to support the expression of inflammatory cytokines in macrophages. To further ensure the specificity of our assay, competitions were performed with the unlabeled C/EBP binding site and an unlabeled CTF/NF-1 binding site. Both with (Fig. 1B) and without (Fig. 1A) LPS treatment, a 100-fold excess of the C/EBP binding site almost completely eliminated detectable C/EBP binding species, while a 100-fold excess of the CTF/NF-1 binding site barely reduced the abundance of such species. Since CRP-1 (C/EBP $\epsilon$ ) (17) has recently been reported to be a myeloid-specific transcription factor (25), we also examined whether this C/EBP family member was present in macrophages. EMSAs did not reveal CRP-1 (C/EBP $\epsilon$ ) binding activity in bone marrow-derived macrophages either before or after LPS stimulation (data not shown).

### Ectopic expression of C/EBP $\alpha$ , C/EBP $\beta$ , and C/EBP $\delta$ in P388 B lymphoblasts

We previously produced two transfectant populations of P388 cells that express C/EBP $\beta$  through a murine retroviral vector (P388-C2 and P388-C2-2) as well as a control population transfected with the same vector lacking an expressed insert (P388-Neo) (9). P388 is a murine B lymphoblastic cell line (15) that lacks C/EBP $\alpha$ , C/EBP $\beta$ , and C/EBP $\delta$  expression (9). To study the capacities of C/EBP $\alpha$  and C/EBP $\delta$  to support the expression of proinflammatory cytokines in comparison to C/EBP $\beta$ , populations of P388 cells were transfected with pSV(X)C/EBP $\alpha$  or pSV(X)C/EBP $\delta$ . Pools of stably transfected cells were obtained after selection with G418. Cells transfected with pSV(X)C/EBP $\alpha$  were designated P388-C $\alpha$ , and cells transfected with pSV(X)C/EBP $\delta$  were designated P388-C $\delta$ . For consistency, the previous P388-C2 cells were designated P388-C $\beta$ .

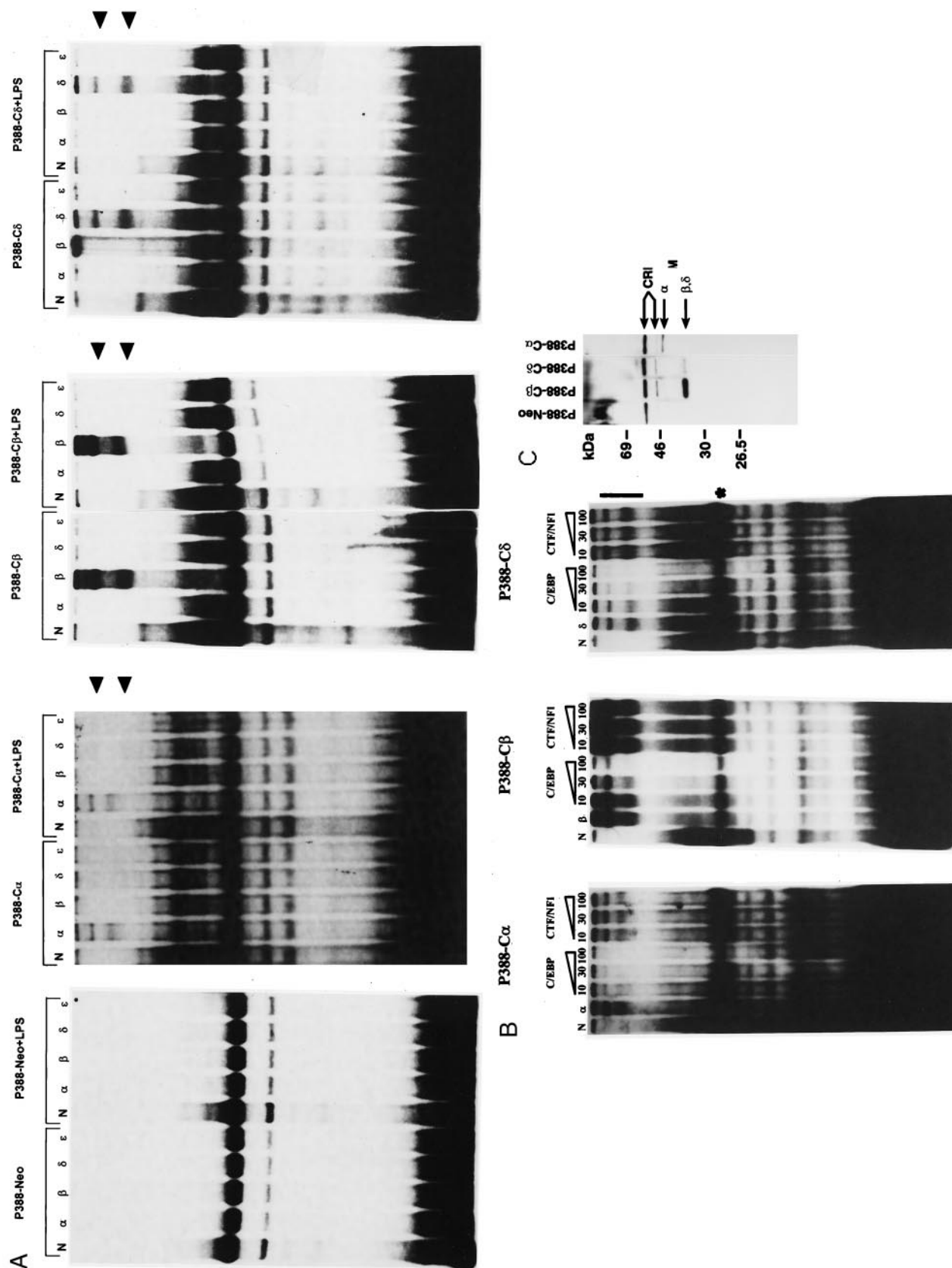


**FIGURE 1.** EMSA of C/EBP $\alpha$ , C/EBP $\beta$ , and C/EBP $\delta$  DNA binding activity in bone marrow-derived macrophages. *A*, No LPS treatment. *B*, Four-hour LPS treatment. Binding reactions included normal rabbit serum (N), C/EBP $\alpha$  antiserum ( $\alpha$ ), C/EBP $\beta$  antiserum ( $\beta$ ), or C/EBP $\delta$  antiserum ( $\delta$ ). Some binding reactions, in addition to specific antisera, included 10-fold (10), 30-fold (30), and 100-fold (100) excess quantities of unlabeled C/EBP or CTF/NF1 binding oligonucleotides. The bar to the right indicates the positions of supershifted EMSA species.

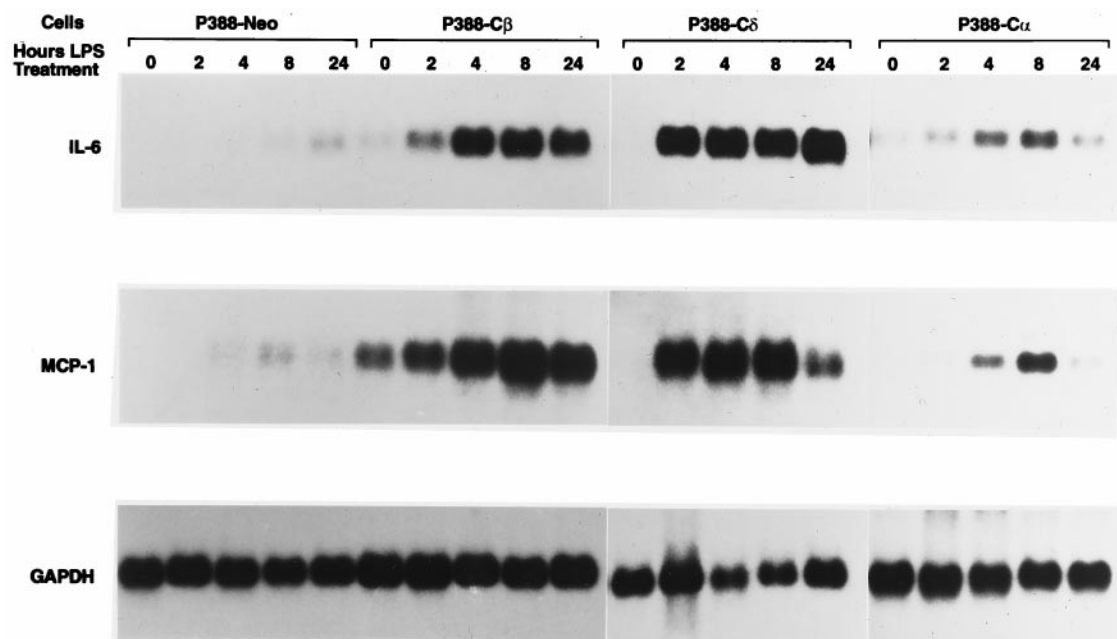
C/EBP expression in the transfected populations was initially characterized by EMSA (Fig. 2A). In comparison to nuclear extracts from P388-Neo, nuclear extracts from P388-C $\beta$ , P388-C $\delta$ , and P388-C $\alpha$  yielded supershifted protein-DNA complexes upon incubation with antisera specific to C/EBP $\beta$ , C/EBP $\delta$ , and C/EBP $\alpha$ , respectively. The EMSA species that gave rise to the supershifts were also evident in the samples incubated with normal rabbit serum. This analysis did not reveal DNA binding activity for any C/EBP family members that had not been transfected into these populations in either the absence or the presence of LPS treatment. Supershift species for C/EBP $\alpha$ , - $\beta$ , and - $\delta$  were only observed in cells transfected for their expression. Additionally,

supershift species for CRP-1 (C/EBP $\epsilon$ ) were not observed in any of the transfectants.

As in the assays using extracts from bone marrow-derived macrophages, competitions were performed with the unlabeled C/EBP binding site and an unlabeled CTF/NF-1 binding site (Fig. 2B). All the supershifted protein-DNA complexes observed upon incubation with antisera specific to C/EBP $\beta$ , C/EBP $\delta$ , and C/EBP $\alpha$  were effectively competed by a 100-fold excess of the C/EBP binding site, while a 100-fold excess of the CTF/NF-1 binding site had little effect. The competition revealed a prominent protein-DNA complex that was not supershifted by specific antisera, but was effectively competed by the unlabeled C/EBP binding site. This



**FIGURE 2.** Analyses of P388 cells stably transfected with C/EBP $\alpha$ , C/EBP $\beta$ , or C/EBP $\delta$  expression vectors. Cell line nomenclature is described in *Results*. **A**, EMSA of C/EBP DNA binding activities in P388 transfectants with and without 4-h LPS treatment. Reactions included normal rabbit serum (N), C/EBP $\alpha$  antiserum ( $\alpha$ ), C/EBP $\beta$  antiserum ( $\beta$ ), C/EBP $\delta$  antiserum ( $\delta$ ), or CRP-1 (C/EBP $\epsilon$ ) antiserum ( $\epsilon$ ). The positions of C/EBP-specific Ab supershift species are indicated by arrowheads on the *right*. **B**, EMSA of C/EBP DNA binding activities in P388 transfectants in the presence of unlabeled competing oligonucleotide. Binding reactions included normal rabbit serum (N), C/EBP $\alpha$  antiserum ( $\alpha$ ), C/EBP $\beta$  antiserum ( $\beta$ ), or C/EBP $\delta$  antiserum ( $\delta$ ). Some binding reactions, in addition to specific antisera, included 10-fold (10), 30-fold (30), and 100-fold (100) excess quantities of unlabeled C/EBP or CTF/NF1 binding oligonucleotides. The bar to the *right* indicates the positions of supershifted EMSA species. The asterisk marks the position of the likely Ig/EBP EMSA species. **C**, Western blot analysis of C/EBP proteins derived from nuclear extracts of the transfectants. The positions of C/EBP $\alpha$  ( $\alpha$ ), C/EBP $\beta$  ( $\beta$ ), C/EBP $\delta$  ( $\delta$ ), and cross-reactive material (CRM) are indicated by arrows on the *right*. The positions of m.w. markers are indicated on the *left*.



**FIGURE 3.** Northern analyses of IL-6 and MCP-1 expression in P388 transfectants. Total RNA was isolated over time courses of LPS treatment as indicated. Ten micrograms of RNA was analyzed on Northern blots that were successively hybridized to probes for IL-6, MCP-1, and GAPDH.

species probably represents Ig/EBP (C/EBP $\gamma$ ), which is highly expressed in P388 cells (data not shown) and other immature B cells (26).

Western blot analysis of nuclear extracts from the same transfected populations using panCRP antiserum confirmed expression of the C/EBPs from the transfected vectors (Fig. 2C). The immunogenic peptide used in generating panCRP antiserum is completely conserved among C/EBP family members (24); thus, this antiserum can be used for quantitative comparisons of protein levels between different C/EBP family members. C/EBP $\beta$  protein levels were much higher than those of C/EBP $\delta$  and C/EBP $\alpha$  (Fig. 2C) even though the abundance of EMSA species among the transfectants, particularly C/EBP $\beta$  and C/EBP $\delta$ , was similar (Fig. 2A). This suggests a higher specific DNA binding activity for C/EBP $\delta$ . Successful transfection of the P388 populations was also confirmed by Southern blot and Northern blot analyses (data not shown).

#### *LPS-induced cytokine expression is supported by C/EBP $\alpha$ and C/EBP $\delta$ as well as C/EBP $\beta$*

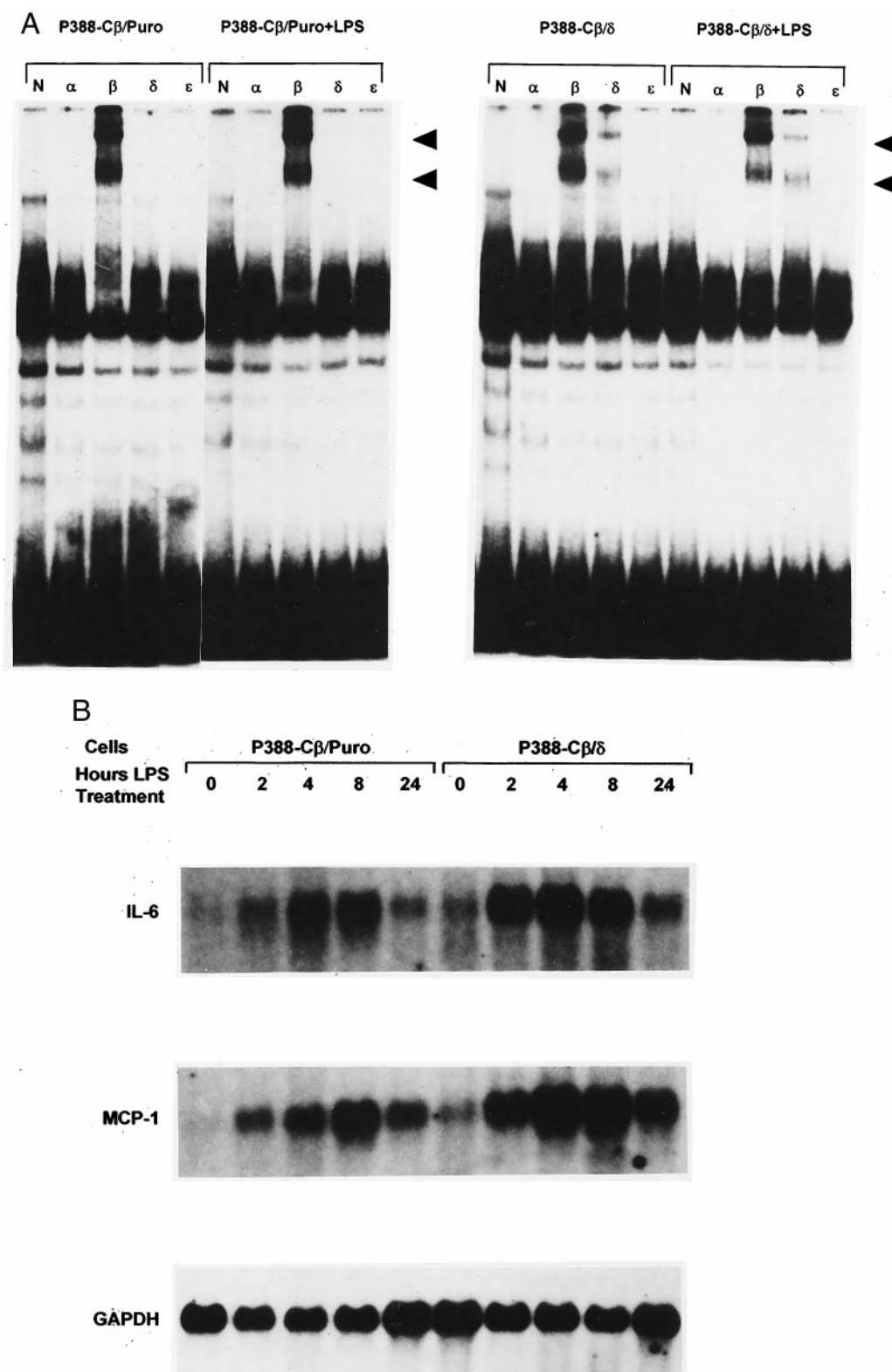
Cultures of P388-C $\beta$ , P388-C $\delta$ , and P388-C $\alpha$  cells were treated with LPS over a time course of 0, 2, 4, 8, and 24 h, and RNA was isolated. A control population of P388 lymphoblasts transfected with pSV(X)Neo was also examined. Northern analyses and RNase protection assays were performed to detect transcripts encoding IL-6, MCP-1, IL-1 $\alpha$ , IL-1 $\beta$ , TNF- $\alpha$ , MIP-1 $\alpha$ , and G-CSF. Transcripts encoding GAPDH were also examined as a normalization control. LPS was found to induce transcripts for IL-6 and MCP-1 in P388-C $\beta$ , P388-C $\delta$ , and P388-C $\alpha$  cells (Fig. 3). All three C/EBPs were quite effective in inducing IL-6 and MCP-1 RNAs. Induction was evident by 2 h of LPS treatment, with a decline by 24 h. The family members differed in the time required to reach peak levels of RNA; C/EBP $\delta$  transfectants required 2 h, and C/EBP $\beta$  and C/EBP $\alpha$  transfectants required as much as 8 h to reach peak levels. C/EBP $\delta$  may also be the most effective family member considering its relatively low abundance in P388-C $\delta$  (Fig. 2B). C/EBP $\alpha$  may be the least effective, as P388-C $\alpha$  cells show

lower peak levels of IL-6 and MCP-1 RNAs. Also, note that C/EBP $\beta$  expression is associated with significantly higher basal levels of MCP-1 transcripts than those seen with either C/EBP $\alpha$  or C/EBP $\delta$  expression. Transcripts encoding IL-1 $\alpha$ , IL-1 $\beta$ , and G-CSF were not induced by LPS (data not shown), and weak LPS inductions of TNF- $\alpha$  and MIP-1 $\alpha$  were not augmented in any of the C/EBP transfectants compared with those in P388-Neo cells (data not shown). These results were reproducible in similar independently transfected populations (data not shown). The various C/EBP family members thus differ subtly in their ability to support cytokine expression.

#### *Coexpression of C/EBP $\delta$ with C/EBP $\beta$ augments the expression of IL-6 and MCP-1, but does not support the expression of additional proinflammatory cytokines*

Since authentic macrophages were demonstrated to express multiple C/EBPs (Fig. 1), we sought to produce transfectants expressing multiple C/EBPs to test whether combinatorial expression confers augmented capacities to transcribe proinflammatory cytokine genes. In particular, we sought to produce cells coexpressing C/EBP $\beta$  and C/EBP $\delta$  because these DNA binding activities were enhanced upon LPS treatment of bone marrow-derived macrophages (Fig. 1). To produce cells expressing both C/EBP $\beta$  and C/EBP $\delta$ , C/EBP $\delta$  was introduced into P388-C $\beta$  cells with the murine retroviral vector pBABE-C/EBP $\delta$ . P388-C $\beta$  cells were transfected with either pBABE-C/EBP $\delta$  or the parental vector lacking an expressed insert, pBABE-Puro. Pools of stably transfected cells were obtained after selection with puromycin. Cells doubly transfected with pSV(X)C/EBP $\beta$  and pBABE-C/EBP $\delta$  were designated P388-C $\beta$ / $\delta$  and cells doubly transfected with pSV(X)C/EBP $\beta$  and pBABE-Puro were designated P388-C $\beta$ /Puro.

Supershifting of EMSA species with specific antisera verified the expression of C/EBP $\beta$  and C/EBP $\delta$  in the doubly transfected population, while the control transfection population expressed only C/EBP $\beta$  (Fig. 4A). Successful transfection was also confirmed by Southern and Northern blot analyses (data not shown). When the LPS induction of IL-6 and MCP-1 RNAs was examined



**FIGURE 4.** Analyses of P388 cells stably transfected for dual expression of C/EBPβ and C/EBPδ. Cell line nomenclature is described in *Results*. *A*, EMSA of C/EBP DNA binding activities in P388 transfectants with and without 4-h LPS treatment. Reactions included normal rabbit serum (N), C/EBPα antiserum (α), C/EBPβ antiserum (β), C/EBPδ antiserum (δ), or CRP-1 (C/EBPε) antiserum (ε). The positions of C/EBP supershift species are indicated by arrowheads on the *right*. *B*, Northern analyses of IL-6 and MCP-1 expression. Total RNA was isolated over time courses of LPS treatment and analyzed as described in Figure 3.

in these transfected populations, the level of expression was augmented in cells expressing both C/EBPβ and C/EBPδ compared with that in cells expressing only C/EBPβ (Fig. 4B). Densitometry

revealed peak inductions of 2.3-fold for IL-6 and MCP-1 in cells expressing C/EBPβ, and peak inductions of 3.5-fold for IL-6 and 3.8-fold for MCP-1 were observed in cells coexpressing C/EBPδ

Table I. Comparison of LPS induction of IL-6 and MCP-1 mRNA between P388-C $\beta$ /Puro and P388-C $\beta$ / $\delta$ <sup>a</sup>

	LPS				
	0	2	4	8	24
P388-C $\beta$ /Puro IL-6 RNA	1.0	1.6	2.3	1.9	0.8
P388-C $\beta$ / $\delta$ IL-6 RNA	1.0	2.8	3.5	3.3	1.5
P388-C $\beta$ /Puro MCP-1 RNA	1.0	1.7	2.1	2.3	1.1
P388-C $\beta$ / $\delta$ MCP-1 RNA	1.0	2.4	3.7	3.8	2.2

<sup>a</sup> The autoradiograms presented in Figure 4B were analyzed by densitometer, and raw values for IL-6 and MCP-1 were normalized to values for GAPDH. The expression levels for each cell line were set at 1.0 at 0 h. LPS treatment and the tabulated values represent fold increases in expression.

and C/EBP $\beta$  (Table I). Whether the coexpression of C/EBP $\delta$  and C/EBP $\beta$  augmented the LPS induction of IL-6 and MCP-1 in an additive or a synergistic manner is unclear. Since the previous data (Figs. 2C and 3) suggest that C/EBP $\delta$  may be more effective than C/EBP $\beta$  in supporting transcription of IL-6 and MCP-1 RNAs, the augmented expression of these mRNAs upon LPS induction may be solely dependent upon the added expression of C/EBP $\delta$ . Examination of IL-1 $\alpha$ , IL-1 $\beta$ , TNF- $\alpha$ , MIP-1 $\alpha$ , and G-CSF expression showed no effect of coexpression of C/EBP $\beta$  and C/EBP $\delta$  on the induction of RNAs encoding these cytokines (data not shown). These results were reproducible in a similar population of P388 cells independently transfected for coexpression of C/EBP $\beta$  and C/EBP $\delta$  (data not shown). Unexpectedly, in repeated attempts we were unable to obtain transfectants coexpressing C/EBP $\alpha$  and C/EBP $\beta$ , or C/EBP $\alpha$  and C/EBP $\delta$ .

#### NF- $\kappa$ B (p50/p65) DNA binding activity is induced by LPS in the P388 transfectants

NF- $\kappa$ B has been implicated in the regulation of numerous cytokines that are expressed by macrophages in response to LPS (reviewed in Refs. 27 and 28). In particular, mutation of an NF- $\kappa$ B binding site in the human IL-6 promoter completely abolished re-

sponsiveness to LPS (29). Additionally, the IL-6 promoter (30) and the IL-8 promoter (30, 31) are activated synergistically by C/EBP $\beta$  and NF- $\kappa$ B. The importance of NF- $\kappa$ B in the expression of proinflammatory cytokines led us to determine whether NF- $\kappa$ B was indeed activated upon LPS treatment of P388 cells. The lack of cytokine induction in P388-Neo cells could be caused by an absence of NF- $\kappa$ B expression or activation. The inability of C/EBP transfectants of P388 cells to induce cytokines other than IL-6 and MCP-1 could be similarly explained. On the other hand, the ability of C/EBP $\beta$  to mediate a higher basal level of MCP-1 expression than other C/EBPs could be caused by constitutive NF- $\kappa$ B activity in P388-C $\beta$  cells. To address these issues, EMSAs were performed using a probe for NF- $\kappa$ B binding. As shown in Figure 5A, an LPS-induced EMSA species was observed in all transfectants, including the P388-Neo control. Formation of this LPS-induced species could be quantitatively blocked by either p50- or p65-specific antisera (Fig. 5B), showing that the major species induced is a p50/p65 heterodimer. Thus, NF- $\kappa$ B (p50/p65) is translocated to the nucleus of P388 cells and is probably available to support the LPS-induced expression of proinflammatory cytokines. The inability of P388 cells to induce IL-6 and MCP-1 can be specifically attributed to the absence of C/EBP family members.

## Discussion

The data presented in this paper demonstrate that C/EBP $\alpha$ , C/EBP $\beta$ , and C/EBP $\delta$  are each sufficient to confer LPS-inducible expression of IL-6 and MCP-1 to P388 B lymphoblasts. We have shown that C/EBP $\alpha$  and C/EBP $\beta$  are expressed in unstimulated bone marrow-derived macrophages, while LPS stimulation down-regulates C/EBP $\alpha$  expression and up-regulates expression of C/EBP $\delta$ . Thus, all three of these C/EBPs are expressed in bone marrow-derived macrophages and could participate in the LPS induction of IL-6 and MCP-1. The observation of a largely normal cytokine response to LPS treatment in the macrophages of C/EBP $\beta$ -deficient mice (11) can be explained by the availability of C/EBP $\alpha$  and/or C/EBP $\delta$ . The induction of C/EBP $\delta$  by LPS in bone marrow-derived macrophages makes it a particularly attractive candidate for replacing C/EBP $\beta$  activity. In fact, C/EBP $\delta$  may be more effective than C/EBP $\beta$  in supporting the transcription of IL-6

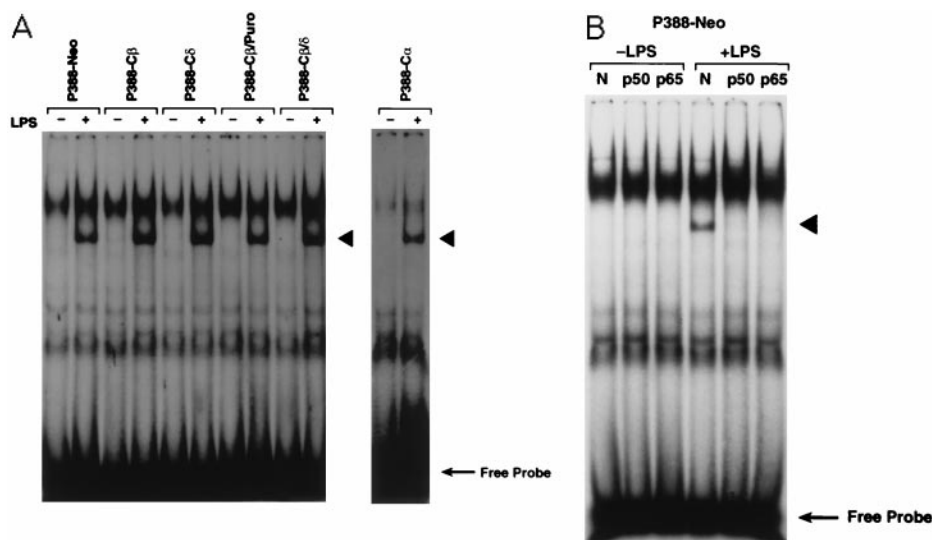


FIGURE 5. EMSA of NF- $\kappa$ B DNA binding activity in P388 transfectants. A, Cells were grown in the absence of LPS (-) or for 4 h in the presence of LPS (+). The position of NF- $\kappa$ B EMSA species is indicated by arrowheads on the right of each panel. B, Nuclear extract of P388-Neo cells was treated with normal rabbit serum (N), p50 antiserum (p50), or p65 antiserum (p65). The arrowhead on the right indicates the position of NF- $\kappa$ B EMSA species.



and MCP-1 genes, since a relatively low level of its expression in P388-C $\delta$  transfectants allows a quite vigorous induction of IL-6 and MCP-1. This induction is at least equal to that observed in P388-C $\beta$  cells, which express a much higher level of C/EBP $\beta$ , and is more rapid. P388-C $\delta$  cells also display a level of DNA binding similar to that of P388-C $\beta$  cells, suggesting a higher sp. act. for DNA binding. C/EBP $\delta$  has previously been reported to be a stronger *trans*-activator than C/EBP $\beta$  using the human IL-6 promoter in a reporter construct (8). The presence of a regulatory domain (RD2) in C/EBP $\beta$  that represses DNA binding activity may explain its lower activity (24). On the other hand, C/EBP $\alpha$  appears less effective than C/EBP $\delta$  in inducing IL-6 and MCP-1 while being expressed at a similar level to C/EBP $\delta$  in transfectants. Additionally, C/EBP $\alpha$  DNA binding activity is reduced upon LPS treatment of bone marrow-derived macrophages, making it a less likely candidate to replace C/EBP $\beta$  activity in C/EBP $\beta$ -deficient mice. Collectively, the data suggest a prominent role for C/EBP $\delta$  in the LPS induction of inflammatory cytokines and implicate C/EBP $\delta$  as the most plausible activity to compensate for the lack of C/EBP $\beta$  in C/EBP $\beta$ -deficient animals.

The kinetics of LPS induction of IL-6 and MCP-1 mRNAs are generally similar among transfectants for the various C/EBP family members. Induction is evident by 2 h and declines by 24 h. There may be differences, however, in the time required to attain peak RNA levels among C/EBP family members. The C/EBP $\delta$  transfectants reached peak levels at 2 h compared with 4 or 8 h for C/EBP $\beta$  and C/EBP $\alpha$  transfectants, and the C/EBP $\beta/\delta$  transfectants showed a dramatic induction by 2 h. Our previous studies (9) found that the kinetics of proinflammatory cytokine mRNA production in a macrophage cell line, P388D1(IL1), also reached peak RNA levels by 2 h. This may suggest the importance of C/EBP $\delta$  expression *in vivo*. Indeed, we have shown in this study that C/EBP $\delta$  is induced in LPS stimulation of bone marrow-derived macrophages. The delay in reaching peak RNA levels for C/EBP $\beta$  and C/EBP $\alpha$  transfectants may indicate a requirement for the induction of other factors for optimal expression with these C/EBPs. The delay may reflect the time required to induce and synthesize these factors, or, on the other hand, the delay may simply indicate a lower rate of transcription requiring longer times to attain peak levels.

It is clear that LPS induction of IL-6 and MCP-1 mRNAs in our system operates through either the post-transcriptional activation of C/EBPs or the induction of a necessary cooperating transcription factor. EMSA analysis demonstrated C/EBP binding activity for the transfected genes before LPS treatment, and LPS treatment neither induced C/EBP family members other than those transfected nor increased the binding activity of the transfected C/EBPs. If LPS treatment is modulating the activity of C/EBPs in our system, it must be in a manner not evident in EMSA analysis. Other investigators have found in transient transfection studies of the IL-6 promoter that coexpression of C/EBP $\beta$  and NF- $\kappa$ B synergistically activates the IL-6 promoter (30), and mutation of an NF- $\kappa$ B binding site in the human IL-6 promoter completely abolished responsiveness to LPS (29). We have found that LPS induces NF- $\kappa$ B (p50/p65) in the P388 transfectants, and it is likely that this is the primary role of LPS in our system.

A synergism between the activities of C/EBP $\beta$  and C/EBP $\delta$  has been reported for the transient *trans*-activation of the human IL-6 promoter (8), and we did observe that coexpression of C/EBP $\delta$  with C/EBP $\beta$  augments the LPS induction of IL-6 and MCP-1 mRNAs over that observed for C/EBP $\beta$  alone. It is unclear from our data whether that augmentation is synergistic or additive, since C/EBP $\delta$  by itself appears more active than C/EBP $\beta$  in supporting LPS induction of IL-6 and MCP-1. Interestingly, despite repeated

attempts we were unable to obtain transfectants doubly expressing C/EBP $\alpha$  and C/EBP $\beta$  or C/EBP $\alpha$  and C/EBP $\delta$ . Although bone marrow-derived macrophages coexpress these C/EBPs, we have not detected C/EBP $\alpha$  expression in any mature macrophage cell lines (our unpublished observation) (9, 13). These observations may indicate that C/EBP $\alpha$  expression is incompatible with the immortalization of mature macrophage cell lines. Consistent with this idea, C/EBP $\alpha$  has previously been shown to inhibit proliferation in adipocytes (32), hepatocytes (33), and other cell types (33).

Among the several cytokine mRNAs examined, only IL-6 and MCP-1 displayed robust LPS inductions. The lack of induction of other cytokines may reflect the requirement of transcription factors in addition to the C/EBP family for a full cytokine response. We have found that NF- $\kappa$ B (p50/p65) is induced by LPS in the P388 transfectants, so such a deficiency must be attributed to other transcription factors. For instance, the murine IL-1 $\beta$  gene requires a novel 6-bp sequence (-2280 to -2275) in addition to C/EBP and NF- $\kappa$ B binding sites (34). Furthermore, previous investigators have noted differences in the regulation of MCP-1, IL- $\alpha$ , and IL-1 $\beta$  (35, 36). For example, agents that elevate intracellular levels of cAMP suppress the LPS induction of MCP-1, but do not affect the induction of IL-1 $\alpha$ , and actually enhance IL-1 $\beta$  induction. Stimuli other than LPS, such as IFN- $\gamma$ , IL-1, or TNF, might also provide a more complete cytokine response through their ability to activate other transcription factors.

An alternative explanation for the lack of induction of cytokines other than IL-6 and MCP-1 may be the expression of Ig/EBP (C/EBP $\gamma$ ) in P388 lymphoblasts. Ig/EBP has been reported to be a *trans*-dominant inhibitor of C/EBP family members (37). It may block C/EBP activity on the promoters of those cytokine genes for which we do not observe activation. It will be of interest to assess the ability of Ig/EBP to inhibit C/EBP activation of the promoters for IL-6 and other proinflammatory cytokines in a transient expression system lacking endogenous Ig/EBP expression.

The expression of IL-6 and MCP-1, while both showing strong inductions with LPS, differ in regard to the basal levels of their mRNAs among the various C/EBP transfectants. In particular, MCP-1 displays an appreciable level of RNA in P388-C $\beta$  in the absence of LPS. Since we do not observe NF- $\kappa$ B activity in the absence of LPS, it appears that MCP-1 does not require NF- $\kappa$ B for significant basal expression of its RNA. It will be of interest to compare the structure of the MCP-1 promoter to that of IL-6.

The data presented here lead us to predict that C/EBP $\delta$  expression may be crucial to supporting proinflammatory cytokine expression *in vivo*. Tanaka et al. (11) did not find severe impairment of proinflammatory cytokine expression in C/EBP $\beta$ -deficient animals. We have now shown that while both C/EBP $\alpha$  and C/EBP $\delta$  can support the LPS activation of endogenous IL-6 and MCP-1 genes, the LPS activation of bone marrow-derived macrophages down-regulates C/EBP $\alpha$  activity and up-regulates C/EBP $\delta$  activity. C/EBP $\delta$  is thus the best candidate for the factor allowing C/EBP $\beta$ -deficient mice to display a largely normal cytokine expression in response to LPS stimulation. A lack of C/EBP $\delta$  expression would be expected to reduce and/or delay peak expression of IL-6 and MCP-1 mRNAs. The development of knockout mice deficient in C/EBP $\delta$  expression and mice deficient in both C/EBP $\beta$  and C/EBP $\delta$  expression should provide the ultimate test of this issue.

Finally, why are there multiple C/EBP family members with seemingly redundant function within the inflammatory response? First, one should recognize that our system has only allowed examination of IL-6 and MCP-1 expression; promoter-specific functions of C/EBP family members are certainly possible for other genes. More significantly, differential function of C/EBP family

members may become apparent under the influence of inflammatory stimuli other than LPS. It is clearly a high priority in future investigations to examine the C/EBP transfectants reported here under conditions of IL-1, IL-6, and TNF stimulation, since differences in function among C/EBP family members may derive from their linkages to different signal transduction pathways.

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