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Dexamethasone Inhibits IL-12p40 Production in Lipopolysaccharide-Stimulated Human Monocytic Cells by Down-Regulating the Activity of c-Jun N-Terminal Kinase, the Activation Protein-1, and NF-κB Transcription Factors[1]

Wei Ma,[‡] Katrina Gee,[†] Wilfred Lim,[†] Kelly Chambers,[†] Jonathan B. Angel,[†] Maya Kozlowski,[§] and Ashok Kumar[2]*†‡

IL-12 plays a critical role in the development of cell-mediated immune responses and in the pathogenesis of inflammatory and autoimmune disorders. Dexamethasone (DXM), an anti-inflammatory glucocorticoid, has been shown to inhibit IL-12p40 production in LPS-stimulated monocytic cells. In this study, we investigated the molecular mechanism by which DXM inhibits IL-12p40 production by studying the role of the mitogen-activated protein kinases (MAPKs), and the key transcription factors involved in human IL-12p40 production in LPS-stimulated monocytic cells. A role for c-Jun N-terminal kinase (JNK) MAPK in LPS-induced IL-12p40 regulation in a promonocytic THP-1/CD14 cell line was demonstrated by using specific inhibitors of JNK activation, SP600125 and a dominant-negative stress-activated protein/extracellular signal-regulated kinase kinase-1 mutant. To identify transcription factors regulating *IL-12p40* gene transcription, extensive deletion analyses of the IL-12p40 promoter was performed. The results revealed the involvement of a sequence encompassing the AP-1-binding site, in addition to that of NF-κB. The role of AP-1 in IL-12p40 transcription was confirmed by using antisense c-*fos* and c-*jun* oligonucleotides. Studies conducted to understand the regulation of AP-1 and NF-κB activation by JNK MAPK revealed that both DXM and SP600125 inhibited *IL-12p40* gene transcription by inhibiting the activation of AP-1 and NF-κB transcription factors as revealed by luciferase reporter and gel mobility shift assays. Taken together, our results suggest that DXM may inhibit IL-12p40 production in LPS-stimulated human monocytic cells by down-regulating the activation of JNK MAPK, the AP-1, and NF-κB transcription factors. *The Journal of Immunology*, 2004, 172: 318–330.

Interleukin-12, a Th1-type cytokine, plays a significant role in the development of cell-mediated immunity against intracellular pathogens including *Mycobacterium tuberculosis*, *Listeria monocytogenes*, and HIV (1–5). IL-12 generates lymphokine-activated killer cell and CTL activity, up-regulates IFN-γ production by NK and T cells, and induces potent antitumor responses in models of local and metastatic tumors (2–4). It facilitates the development of Th1-type responses and inhibits differentiation of Th2 cells and Th2-type responses, and has been implicated in the pathogenesis of infectious, inflammatory, and autoimmune diseases such as multiple sclerosis and experimental autoimmune encephalomyelitis (3–8). IL-12 is produced by monocytes/macrophages, B cells, dendritic cells, and possibly other accessory cells (1–5). IL-12 is a heterodimeric 70-kDa glycoprotein composed of two distinct disulfide-linked subunits that have molecular masses of 35 and 40 kDa, respectively. Transcripts for the *IL-12p35* gene have been detected in almost all cell types tested including hemopoietic and tumor cell lines (9, 10). In contrast, expression of IL-12p40 is strictly regulated, and IL-12p40 transcripts are detected only in cell types that produce biologically active IL-12 (9, 10). Therefore, levels of IL-12p40 are a better indicator of IL-12 production, and as a result, the *IL-12p40* gene has been studied for transcriptional regulation.

Because IL-12 plays a critical role in host defense, understanding the regulation of IL-12 expression, especially that of the inducible IL-12p40 component, and characterization of the signal transduction events involved, may lead to the development of strategies for the treatment of autoimmune diseases and cancer. LPS, a bacterial cell wall component, is the best-characterized monocytic mitogen and acts as a potent inducer of proinflammatory and immunoregulatory cytokines including IL-12 (11, 12). There is relatively little information on the role of intracellular signaling molecules that regulate IL-12 synthesis in human monocytic cells following LPS stimulation. LPS-induced cell signaling involves

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[3] Abbreviations used in this paper: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; hIL-12p40, human IL-12p40; DXM, dexamethasone; DN, dominant negative; MKK4, MAPK kinase 4; SEK1, stress-activated protein/Erk kinase-1; wt, wild type; m, mutant; GRE, glucocorticoid response element.

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activation of tyrosine and serine/threonine protein kinases including protein kinase C, and the mitogen-activated protein kinases (MAPKs)[3] (13–18). In this study, we focused on the role of the MAPKs in IL-12 production, because these signaling molecules play a key role in lymphocytic proliferation, differentiation, and apoptosis (15, 19). The MAPKs include p38, the extracellular signal-regulated kinases (ERKs), and the c-Jun N-terminal kinase (JNK). These three families of MAPKs form three parallel signaling cascades activated by distinct and sometimes overlapping sets of stimuli (13, 15, 17). In general, ERKs are activated by growth factors, whereas the p38 and JNK are activated by stress stimuli (15).

IL-12 expression is regulated at the level of transcription, and multiple transcription factors and their complexes have been suggested to play a key role in IL-12p40 regulation in human and murine monocytic cells (20–27). Studying the mouse promoter, Murphy et al. (24) provided evidence for the role of NF-κB transcription factors in IL-12p40 regulation in IFN-γ-stimulated monocytic cells. Subsequently, the C/EBP transcription factor, in cooperation with the rel/NF-κB complex, was found to regulate the murine and human *IL-12p40* gene (21). In contrast, Ma et al. (22, 23) demonstrated the role of the Ets-2 transcription factor in stimulation of human IL-12p40 (hIL-12p40) promoter in IFN-γ- and LPS-stimulated murine RAW264 monocytic cells. Recently, several other studies have reported the involvement of IFN-γ regulatory factors, NF-κB, Ets-2, and PU.1 transcription factors in IL-12p40 regulation (25, 27).

Dexamethasone (DXM), a potent anti-inflammatory and immunosuppressive glucocorticoid, is widely used in the treatment of inflammation, allergic diseases, and a number of autoimmune disorders (28). The immunosuppressive properties of glucocorticoids have been ascribed to their ability to suppress the synthesis of a number of cytokines including that of IL-12p40 (29–31). The molecular mechanism by which DXM inhibits IL-12p40 production remains unknown. The glucocorticoids have been shown to mediate their biological effects on cytokine production primarily by down-regulating JNK MAPK activation (32, 33) as well as AP-1 and NF-κB activity (34–39). Therefore, we investigated the role of MAPKs and critical transcription factors in IL-12p40 regulation in LPS-stimulated normal human monocytic cells and in promonocytic THP-1 cells following treatment with DXM. The results revealed that p38 and ERK MAPKs did not regulate IL-12p40 production in purified normal human monocytes. In contrast, DXM treatment significantly inhibited LPS-induced IL-12p40 production by normal monocytes and THP-1 cells, suggesting a role for JNK MAPK and/or the AP-1 transcription factor in IL-12p40 regulation. The role of JNK was confirmed by using specific inhibitors of JNK, SP600125 (40), and a dominant-negative (DN) mutant of the stress-activated protein/Erk kinase-1 (SEK1), which is upstream of JNK in the MAPK pathway. To understand the role of the AP-1 transcription factor, we generated a panel of IL-12p40 promoter deletion mutants fused to the luciferase reporter gene and examined the ability of these promoter fragments to drive the expression of the luciferase gene in LPS-stimulated THP-1 cells. The results of these analyses suggest a previously unrecognized role for AP-1 in addition to that of NF-κB in the regulation of the *hIL-12p40* gene. The involvement of AP-1 in IL-12p40 transcription was further confirmed by interfering with the IL-12p40 production in cells using antisense c-*fos* and c-*jun* oligonucleotides. The data presented in this report also suggest the involvement of JNK in IL-12p40 production through the activation of AP-1 and NF-κB transcription factors in LPS-stimulated human monocytic cells.

Materials and Methods

Isolation of monocytes from PBMCs

PBMCs were isolated from the blood of healthy adult volunteers following approval of the protocol by the Ethics Review Committee of the Children's Hospital of Eastern Ontario. PBMCs were isolated by density gradient centrifugation over Ficoll-Hypaque (Amersham Biosciences, Piscataway, NJ). Purified monocytes were isolated from the PBMCs as described previously (41). Briefly, the cell layer containing mononuclear cells was collected and washed three times in PBS. Purified, nonactivated monocytes were isolated by negative selection by depletion of T cells and B cells using magnetic polystyrene Dynabeads coated with Abs specific for CD2 (T cells) and CD19 (B cells) (Dynal Biotech, Oslo, Norway), as described earlier (18). Briefly, PBMCs ($10–20 \times 10^6$ cells/ml) were resuspended with Dynabeads M-450 pan T (CD2) and pan B (CD19) for 30 min on ice with constant rocking. $CD2^+CD19^+$ cells were separated magnetically from the $CD2^-CD19^-$ cells. $CD2^-CD19^-$ cells were incubated at 37°C for 2 h following which nonadherent cells were removed. The adherent mononuclear cells obtained contained <1% $CD2^+$ T cells and $CD19^+$ B cells as determined by flow-cytometric analysis.

Cell lines, cell culture, and reagents

THP-1, a promonocytic cell line derived from a human acute lymphocytic leukemia patient, was obtained from the American Type Culture Collection (Manassas, VA). Five to 15% of THP-1 cells express CD14, and following LPS stimulation, the levels of CD14 expression is increased to ~50% (42). THP-1 cells transfected with a plasmid containing CD14 cDNA sequences (THP-1/CD14) were kindly provided by Dr. R. Ulevitch (The Scripps Research Institute, La Jolla, CA) (42). Cells were cultured in IMDM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS (Invitrogen/Life Technologies, Grand Island, NY), 100 U/ml penicillin, 100 μg/ml gentamicin, 10 mM HEPES, and 2 mM glutamine. PD98059, an inhibitor of mitogen-activated protein/ERK kinase-1 kinase that selectively blocks the activity of ERK MAPK (15, 43) was purchased from Calbiochem (San Diego, CA). The pyridinyl imidazole, SB202190, a potent and specific inhibitor of p38 MAPK (15, 16) was also purchased from Calbiochem. SP600125, a specific JNK inhibitor (BIOMOL, Plymouth Meeting, PA), is a reversible ATP competitive inhibitor with >300-fold selectivity vs related MAPKs including ERK1 and p38 as well as protein kinase A and IκB kinase-2 (40). DXM (Sabex, Boucherville, Quebec, Canada), LPS derived from *E. coli 0111:B4* (Sigma-Aldrich), and human rIL-10 (R&D Systems, Minneapolis, MN) were also purchased. All other chemicals used for Western blotting were obtained from Sigma-Aldrich.

Cell stimulation and collection of supernatants

To determine the effects of the p38, p42/44, and JNK MAPK inhibitors, and of DXM on IL-12p40 production, purified monocytes (1×10^6 cells/ml) and THP-1 cells (0.5×10^6 cells/ml) were incubated in 24-well culture plates (Falcon; BD Biosciences, Franklin Lakes, NJ). Cells were left untreated or stimulated with LPS (1 μg/ml) for 48 h in the presence or the absence of MAPK inhibitors. Cell supernatants were frozen at −70°C and thawed at the time of analysis for IL-12p40 production by ELISA.

Antisense oligonucleotides

c-*jun* and c-*fos* antisense oligonucleotides were purchased from Synthegen (Houston, TX). The oligonucleotide sequences are as follows: c-*jun*, 5′-TGC AGT CAT AGA AC-3′; c-*fos*, 5′-GAA GCC CGA GAA CAT CAT-3′; and control, 5′-ATG AGT TTC TCG GGC TTG-3′. The cells were incubated with the c-*jun* and c-*fos* antisense oligonucleotides at different concentrations (1–10 μM) for 4 h followed by stimulation with LPS (0.1–0.5 μg/ml). The culture supernatants were harvested 48 h after stimulation for measurement of IL-10 and IL-12p40 production by ELISA.

Measurement of IL-10 and IL-12p40 production

IL-10 and IL-12p40 were measured in the culture supernatant by ELISA using two different mAbs that recognize distinct epitopes as described previously (41). Briefly, the plates (Immunomodules; Nunc, Roskilde, Denmark) were coated overnight at 4°C with the primary Ab (anti-IL-10 mAb from BD PharMingen (San Diego, CA) at a final concentration of 5 μg/ml; anti-IL-12p40 mAb from R&D Systems at a final concentration of 4 μg/ml) in coating buffer (0.04 M Na_2CO_3, 0.06 M $NaHCO_3$ (pH 9.6)). The plates were washed with PBS-Tween 20 and blocked with PBS-10% FBS. The cytokines were detected by using a second biotinylated mAb in PBS-10% FBS (anti-IL-10 mAb from BD PharMingen at a final concentration of 4 μg/ml; anti-IL-12p40 mAb from R&D Systems at a final concentration of 350 ng/ml). Streptavidin-peroxidase (Jackson ImmunoResearch, West

Grove, PA) was used at a final dilution of 1/1000. The color reaction was developed by *o*-phenylenediamine (Sigma-Aldrich) and hydrogen peroxide and was read at 450 nm. rIL-10 and rIL-12p40 (R&D Systems) were used as standards. The sensitivity of the ELISA for IL-10 and IL-12p40 was 16 pg/ml. Measurement of IL-12p40 was considered to be equivalent to the measurement of dimeric IL-12, because p40 is the inducible subunit.

RNA isolation and semiquantitative RT-PCR for IL-12p40

Total RNA was extracted as described using a monophasic solution containing guanidine thiocyanate and phenol (Tri Reagent solution; Molecular Research Center, Cincinnati, OH) (41). Total RNA (1 μg) was reverse transcribed by using Moloney murine leukemia virus reverse transcriptase (PerkinElmer, Foster City, CA). Equal aliquots (5 μl) of cDNA equivalent to 100 ng of RNA were subsequently amplified for IL-12p40 and β-actin. The oligonucleotide primer sequences for IL-12p40 and β-actin (Stratagene, La Jolla, CA) were as follows: IL-12p40, sense, 5′-GGA CCA GAG CAG TGA GGT CTT-3′; IL-12p40, antisense, 5′-CTC CTT GTT GTC CCC TCT GA-3′; β-actin, sense, 5′-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3′; and β-actin, antisense, 5′-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3′. The amplification conditions for IL-12p40 and β-actin were as follows: denaturation at 94°C for 30 s, annealing at 58°C for 1 min, and extension at 72°C for 2 min. After 30 cycles, the amplified products IL-12p40 (373 bp) and β actin (663 bp) were resolved by electrophoresis on 1.2% agarose gels and visualized by ethidium bromide staining.

Western blot analysis

Phosphorylation of p38, p42/44, and JNK MAPK was determined by Western blot analysis using the corresponding MAPK-specific Abs as described earlier (42). Briefly, cells were treated with varying concentrations of MAPK inhibitors for 2 h before stimulation at 37°C for 0–15 min with LPS (1 μg/ml). Cells were then placed at 4°C and washed with ice-cold PBS. Cell pellets were lysed for 30 min with lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 100 mM NaF, 100 mM sodium orthovanadate, and 1 mM EGTA (pH 7.7)), followed by centrifugation for 20 min at 14,000 $\times$ *g* at 4°C. Protein concentration in supernatants was determined using the Bio-Rad protein assay kit (Bradford method; Bio-Rad, Hercules, CA). Equal amounts of crude cell proteins were subjected to electrophoresis on 8% polyacrylamide-SDS gels. The proteins were transferred onto polyvinylidene difluoride membranes (Pall Gelman Laboratory, Ann Arbor, MI) and were probed with the rabbit anti-human phospho-p38 (New England Biolabs, Mississauga, Ontario, Canada), mouse anti-human phospho-p42/44 or rabbit anti-human phospho-JNK Ab (Santa Cruz Biotechnologies, Santa Cruz, CA), followed by goat anti-mouse or goat anti-rabbit polyclonal Abs conjugated to HRP (Bio-Rad). The membranes were stripped of the primary Abs and reprobed with Abs specific for each of the unphosphorylated p38, p42/44, and JNK MAPKs, as described (42). The immunoblots were visualized by ECL (Amersham Biosciences).

Construction of luciferase reporter gene vectors

Luciferase reporter gene vectors containing IL-12p40 promoter fragments were constructed as described earlier (42). A series of hIL-12p40 promoter fragments (see Fig. 5*A*; fragment −880 to +108; GenBank accession no. U89323) were amplified from genomic DNA by PCR. The primers with restriction sites used to amplify the hIL-12p40 promoter fragments from genomic DNA are shown in Table I. The amplification consisted of denaturation at 95°C for 2 min followed by 30 cycles of the following: denaturation at 95°C for 30 s, annealing at 59°C for 2 min, and extension at 72°C for 2 min, and final elongation at 72°C for 10 min. The amplified promoter products were subcloned into the *Nhe*I/*Nco*I polylinker site of the basic luciferase reporter plasmid, pGL3B, and sequences were confirmed again. To introduce mutations in various transcription factor binding sites within hIL-12p40 promoter, site-directed mutagenesis was performed by PCR using mutagenic primers. The substitutive (site-directed) mutations (−232 to +108), including AP-1 binding sequence (wild type (wt), TTATTCC; mutant (m), ttttccc), Ets-2 binding sequence (wt, TTTCCT; m, ggacct), PU.1 binding sequence (wt, AAGGAA; m, ttcgaa), and NF-κB binding sequence (wt, TTGAAATTCCCC; m, tgggttttgccc), were generated (Table I) (see Fig. 6). The lowercase letters indicate mutated oligonucleotides. The fragments containing these mutations then were inserted into the pGL3B reporter vector. The DNA sequencing was performed by the Biotechnology Research Institute, University of Ottawa.

Transient transfection

THP-1/CD14 cells were transfected with plasmids containing the various IL-12p40 promoter fragments using the Lipofectamine reagent (Invitrogen/Life Technologies) according to the manufacturer's instructions and as described earlier (42). Six micrograms of the test plasmid and 3 μg of the pSV-β-galactosidase internal control vector (Promega, Madison, WI) were incubated for 45 min at room temperature with 10 μl of Lipofectamine reagent in 200 μl of Opti-MEM I reduced serum medium (Invitrogen/Life Technologies) to allow formation of DNA-liposome complexes. These complexes were added to the cell suspension in each well, and cells were cultured for 24 h. Following incubation, cells were harvested and then assayed for luciferase and β-galactosidase activity by using Luciferase assay and β-galactosidase assay kits (both from Promega) in a Bio Orbit 1250 luminometer (Fisher, Pittsburgh, PA). THP-1/CD14 cells were also transfected with either a pcDNA-3 plasmid expressing a DN mutant of MAPK kinase 4 (MKK4)/SEK1 (provided by Dr. J. Woodget (Princess Margaret Hospital, Toronto, Ontario, Canada)) or a control pcDNA-3 plasmid using the above-mentioned protocol and as described earlier (18).

EMSAs

Gel mobility assays were performed as per the standard technique and as described earlier (42). Cells (10^7) were harvested in Tris-EDTA-saline buffer (pH 7.8) and centrifuged at 200 $\times$ *g* for 5 min at 4°C. The cells were lysed for 10 min at 4°C with buffer A (10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, and 0.5 mM PMSF (pH 7.9)) containing 0.1% Nonidet P-40. The lysates were centrifuged at 20,000 $\times$ *g* for 10 min at 4°C. The pellet containing the nuclei was suspended in buffer B (20 mM HEPES, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 25% glycerol) at 4°C for 15 min. Both buffers A and B contained the proteolytic inhibitors including DTT, PMSF, and spermidine at concentrations of 0.5 mM each, as well as 0.15 mM spermine, and 5 μg/ml each of aprotinin, leupeptin, and pepstatin. The supernatant containing the nuclear proteins was collected and frozen at −80°C. Nuclear proteins (5 μg) were mixed for 20 min at room temperature with either ^{32}P-labeled AP-1 or NF-κB oligonucleotide probes, and the complexes were subjected to nondenaturing 5% PAGE for 90 min. The oligonucleotide sequences corresponding to the NF-κB and AP-1 binding sites in the IL-12p40 promoter were as follows: NF-κB, 5′-AGG AAC TTC TTG AAA TTC CCC CAG AAG GTT TT-3′ and 3′-TCC TTG AAG AAC TTT AAG GGG GTC TTC CAA AA-5′;

Table I. *Primer sequences used to amplify the hIL-12p40 promoter fragments from genomic DNA*

Primer Name	Primer Sequence	Region Amplified (bp)	Product Length (bp)
Sense primers			
IL-12 PromC/E	5′-AGGCTAGCCAATGTTGCAACAAGTCAG-3′	−84/+108	192
IL-12 PromNFM	5′-AGGCTAGCCTTCTTGGTTTTGCCCCAGAAGG-3′	−120/+108	228
IL-12 PromNF	5′-AGGCTAGCCTTCTTGAAATTCCCCCAGAAGG-3′	−120/+108	228
IL-12 PromEPNM	5′-AGGCTAGCATATTCCCCACCCAAAAGTCAGGACCTCT-3′	−232/+108	340
IL-12 PromAPM	5′-ACGCGTGCTAGCTTTTCCCCCACCCAAAAG-3′	−232/+108	340
IL-12 PromAP	5′-TGGCTAGCTTATTCCCCACCCAAAAG-3′	−232/+108	340
IL-12 PromSp	5′-AAGCTAGCTGACCGCCCCTTGGCCTTC-3′	−358/+108	466
IL-12 PromNFIL	5′-TGGCTAGCTGTTTCTGAAATTAAAGGAC-3′	−621/+108	724
IL-12 PromIRF	5′-AGGCTAGCGGGTATTTCACTTTCTGCTCC-3′	−733/+108	841
IL-12 Prom	5′-TGGCTAGCCCACGAGATATC-3′	−880/+108	988
Antisense primer	5′-ATTCCATGGCTGCAGGCCCACAGGGAG-3′		

AP-1, 5′-TCC TTC CTT ATT CCC CAC CCA-3′ and 3′-AGG AAG GAA TAA GGG GTG GGT-5′. The mutant oligonucleotide sequences used as cold competitors corresponding to the NF-κB and AP-1 binding sites were as follows: NF-κB (m), 5′-AGG AAC TTC TT**c** **cc**A TTC CCC CAG AAG GTT TT-3′ and 3′-TCC TTG AAG AAg **ggT** AAG GGG GTC TTC CAA AA-5′; AP-1, 5′-TCC TTC CT**g** A**c**T **tg**C CAC CCA-3′ and 3′-AGG AAG GA**c** T**g**A **ac**G GTG GGT-5′. To illustrate specificity of NF binding for NF-κB and AP-1 probes, parallel EMSA reactions were incubated with 50- to 200-fold excess of cold unlabelled probe. Subsequently, supershift analyses were also performed to identify the transcription factors NF-κB and AP-1 by using specific mouse anti-NF-κB p50 and p65 mAbs (Santa Cruz Biotechnologies) and rabbit anti-c-*fos* (Upstate Biotechnology, Lake Placid, NY) and rabbit anti-c-*jun* (Santa Cruz Biotechnologies) polyclonal Abs, respectively. Briefly, nuclear extracts were incubated with the NF-κB oligonucleotides in the presence of anti-NF-κB p50 or p65 Abs, anti-c-*fos*, anti-c-*jun* Abs, or control Abs at a final concentration of 20 μg/ml. The bound and unbound ^{32}P-labeled oligonucleotides were resolved by gel electrophoresis as described above. The gel was dried and exposed to x-ray film (Kodak, Rochester, NY).

Statistical analysis

Means were compared by two-tailed Student's *t* test. The results are expressed as mean ± SEM.

Results

LPS-induced IL-12p40 production by normal human monocytes does not involve the activation of either p38 or p42/44 ERK MAPKs

LPS has been shown to be a potent monocytic mitogen for IL-12p40 production (44, 45). We confirmed these observations and show that purified normal human monocytes secrete IL-12p40 in response to LPS stimulation (Fig. 1*B*). To investigate the role of MAPKs in LPS-induced IL-12p40 production, we first examined the activation of ERK and p38 MAPKs in normal human monocytes. Purified monocytes freshly isolated from healthy individuals were stimulated with LPS for 15 min and subjected to Western immunoblotting for p38 and ERK activation by using anti-phospho-p38 and anti-phospho-p42/44 ERK-specific Abs, respectively. The same blots were stripped and reprobed with anti-p38 and anti-42/44 Abs to ensure equal protein loading. The results show that LPS stimulation induced the phosphorylation of p38 and p42/44 ERKs (Fig. 1*A*). To delineate the role of distinct members of the MAPK family involved in the regulation of LPS-induced IL-12p40 production, we used specific inhibitors of p38 (SB202190) and p42/44 ERKs (PD98059). To determine whether SB202190 and PD98059 inhibited the phosphorylation of p38 and ERKs, respectively, monocytes were treated with these inhibitors at varying concentrations ranging from 0 to 50 μM for 2 h followed by stimulation with LPS for 10 min. The results confirmed our earlier observations that both SB202190 and PD98059, at concentrations of 10 μM, inhibited the phosphorylation of p38 and ERKs, respectively (18) (Fig. 1*A*).

To determine the role of p38 and p42/44 MAPKs, we analyzed IL-12p40 production in LPS-stimulated monocytes treated with the specific inhibitors of p38 and p42/44 ERKs. For this, purified monocytes were treated with SB202190 and PD98059 for 2 h before stimulation with LPS for 48 h. IL-12p40 production was not inhibited by either SB202190 or PD98059 at any concentration (Fig. 1*B*). Doses >50 μM for these inhibitors were not used because these concentrations were cytotoxic as determined by the trypan blue exclusion test. These results suggest that LPS-induced IL-12p40 production in normal monocytes does not involve the activation of either p38 or p42/44 ERKs. In fact, SB202190 enhanced IL-12p40 production (Fig. 1*B*). We have previously shown that treatment of monocytes with SB202190 inhibits LPS-induced IL-10 production (42). The modest but reproducible increase in IL-12p40 production following treatment of monocytes with SB202190 may be attributed to the decreased endogenous production of IL-10.

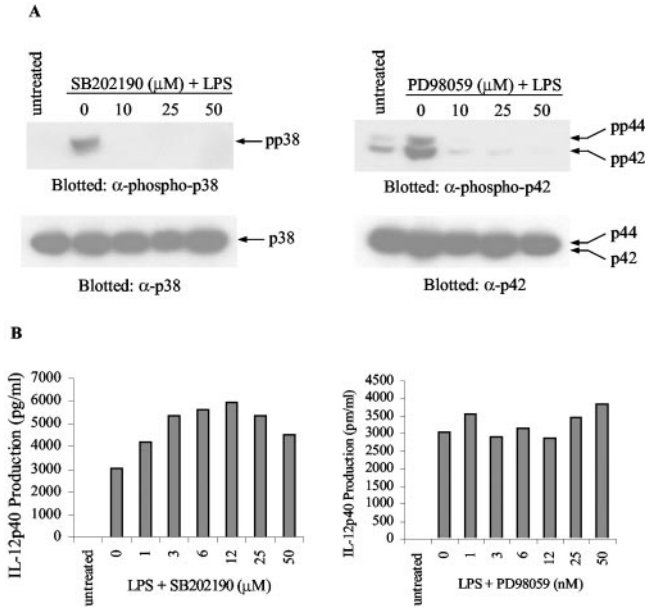

FIGURE 1. p38 and p42/44 MAPK inhibitors do not affect LPS-induced IL-12p40 production in human monocytes. *A*, Purified normal human monocytes (1.0×10^6/ml) were pretreated with either SB202190 or PD98059 at varying concentrations ranging from 0 to 50 μM for 2 h before LPS (1 μg/ml) stimulation for 10 min. Total proteins (50 μg) were subjected to SDS-PAGE followed by Western blot analysis using either anti-phospho-p38 (pp38) or anti-phospho-p42/44 (indicated by arrows as pp42/44) Abs. To control for equal loading of proteins, the membranes were stripped and reprobed with either anti-p38 or anti-p42/44 Abs, respectively (indicated by arrows as p38 and p42/44). *B*, Cells were treated with varying concentrations of inhibitors ranging from 0 to 50 μM for 2 h before stimulation with LPS (1 μg/ml). The supernatants were harvested after 48 h and analyzed by ELISA for IL-12p40 production. The results shown are representative of five independent experiments performed.

DXM inhibits IL-12p40 production in LPS-stimulated monocytes and THP-1/CD14 cells

The lack of involvement of p38 and p42/44 ERKs in LPS-induced IL-12p40 production prompted us to examine the role of JNK, the third major member of the MAPK family. To determine whether the JNK signaling pathway was involved in IL-12p40 production, we took advantage of the fact that glucocorticoids inhibit the activation of JNK (32, 33). We examined whether LPS could induce JNK phosphorylation in normal human monocytes, and whether DXM could inhibit this phosphorylation. Monocytes were treated with DXM at varying concentrations for 2 h before stimulation with LPS. The results support our earlier observations and show that LPS-induced JNK phosphorylation in normal monocytes was inhibited by DXM in a dose-dependent manner (18) (Fig. 2*A*). LPS-induced IL-12p40 production was inhibited by DXM at very low concentrations of 15 nM (Fig. 2*A*).

To understand the molecular mechanism, and specifically the role of JNK in LPS-induced IL-12p40 production in human monocytic cells, we used the promonocytic THP-1/CD14 cell line which constitutively expressed LPS receptor, CD14, on their surface membrane (42) (data not shown). Stimulation of THP-1/CD14 cells with LPS induced high levels of IL-12p40 production as determined by ELISA and RT-PCR analysis (Fig. 2, *B* and *C*). To assess the involvement of JNK, we examined whether LPS could

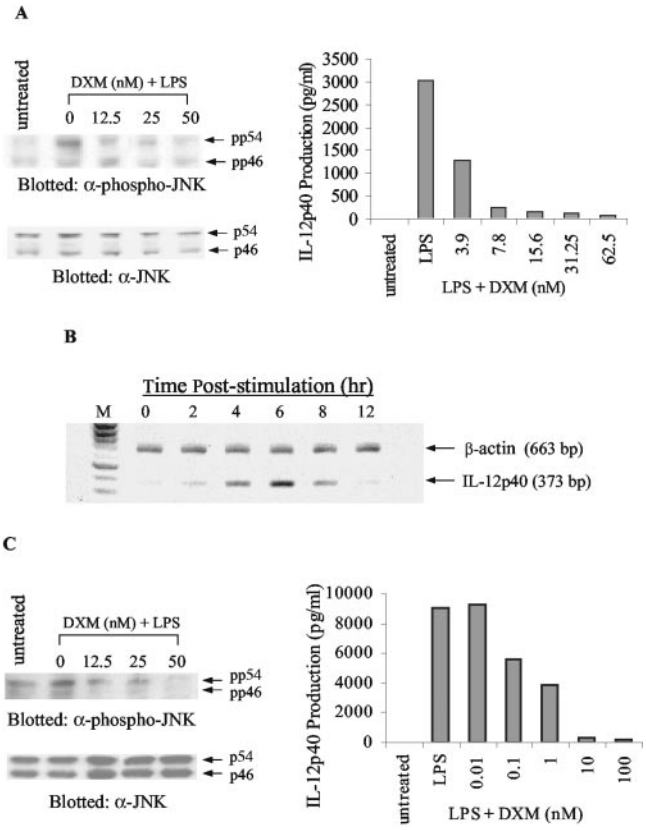

FIGURE 2. DXM inhibits LPS-induced IL-12p40 production in normal monocytes and THP-1/CD14 cells. *A*, *Left panel*, Normal monocytes (1.0×10^6/ml) were pretreated with DXM at concentrations ranging from 0 to 50 nM for 2 h before LPS (1 μg/ml) stimulation for 15 min. Total proteins (50 μg) were subjected to SDS-PAGE followed by Western blot analysis using anti-phospho-JNK1 Abs (indicated by arrows as pp46 and pp54). To control for equal loading, the membrane was stripped and reprobed with anti-JNK Abs (indicated by arrows as p46 and p54). *Right panel*, Monocytes were pretreated with DXM at concentrations ranging from 0 to 62.5 nM for 2 h before LPS (1 μg/ml) stimulation for 48 h, following which cell supernatants were harvested and analyzed for IL-12p40 production. The effects of DXM on IL-12p40 production were qualitatively similar in seven different donors. *B*, THP-1/CD14 cells were stimulated with LPS (1 μg/ml) for various times ranging from 2 to 12 h. Cells were harvested for mRNA isolation, and IL-12p40 expression was determined by semiquantitative RT-PCR analysis using β-actin as a standard control. The results shown are representative of three independent experiments performed. *C*, *Left panel*, THP-1/CD14 cells were pretreated with DXM at concentrations ranging from 0 to 50 nM for 2 h before LPS (1 μg/ml) stimulation for 15 min. Total proteins (50 μg) were subjected to SDS-PAGE followed by Western blot analysis. The membranes were blotted with anti-phospho-JNK1 Ab, and to control for protein loading, the membrane was stripped and reprobed with anti-JNK Ab. *Right panel*, THP-1/CD14 cells were pretreated with DXM at concentrations ranging from 0 to 100 nM for 2 h before LPS (1 μg/ml) stimulation for 48 h, following which cell supernatants were harvested and analyzed for IL-12p40 production. The results shown are representative of three independent experiments performed.

induce JNK phosphorylation in THP-1/CD14 cells in a manner similar to normal monocytes, and whether DXM could inhibit its phosphorylation. LPS induced phosphorylation of JNK, and this activation was inhibited in a dose-dependent manner by DXM (Fig. 2*C*). LPS-induced IL-12p40 production in THP-1/CD14 cells was also highly sensitive to DXM in a manner similar to that of monocytes (Fig. 2*C*).

JNK MAPK plays a distinct role in LPS-induced IL-12p40 production in THP-1 cells

JNK is a cellular target of the SEK1 kinase and expression of the DN SEK1 interferes with JNK phosphorylation by competing out endogenous SEK1 (46, 47). To confirm the role of the JNK pathway in IL-12p40 production, THP-1/CD14 cells were transfected with either a plasmid expressing a DN SEK1 kinase mutant or with a control plasmid (pcDNA3). Cells transfected for 12 h with either the DN SEK1 or the control plasmids, were stimulated with LPS and analyzed by ELISA for IL-12p40 production. The period of 12 h posttransfection before LPS stimulation was identified as the optimal time following cell stimulation with LPS for different time periods (data not shown). IL-12p40 production was significantly reduced in DN SEK1-transfected cells compared with the cells transfected with the control plasmid (Fig. 3*A*; $p < 0.05$). The effect of transfection of THP-1/CD14 cells with DN SEK1 on IL-12p40 production was selective, because the expression of CD14, and the IL-10 production following LPS stimulation remained unaffected (18) (data not shown). Recently, a specific JNK inhibitor, SP600125, has become commercially available (40). To confirm the involvement of JNK, THP-1/CD14 cells were pretreated with varying concentrations of SP600125 before stimulation with LPS. SP600125 inhibited JNK phosphorylation induced by LPS in a dose-dependent manner (Fig. 3*B*). To confirm the specificity of SP600125, the same blots were stripped and probed with either anti-phospho p38 or anti-phospho p42/44 Abs. The results show that SP600125 did not inhibit the phosphorylation of either p38 or p42/44 MAPKs (Fig. 3*B*). Consistent with the results obtained with DXM, SP600125 inhibited LPS-induced IL-12p40 production in a dose-dependent manner (Fig. 3*B*). Furthermore, both DXM (data not shown and Ref. 18) and SP600125 did not affect LPS-induced IL-10 production (263 ± 11 vs 235 ± 54 pg/ml). These results suggest that JNK MAPK activation may be involved in LPS-induced IL-12p40 production in normal human monocytic cells.

Analysis of the IL-12p40 promoter region required for IL-12p40 transcription

The hIL-12p40 promoter has been characterized (23, 24). To understand the regulation of *IL-12p40* gene transcription in LPS-stimulated THP-1/CD14 cells, we used PCR to clone the IL-12p40 promoter fragment encompassing nucleotide residues from 5′ −880 to 3′ +108 bp relative to the +1 transcription start site (Fig. 4). The amplified promoter fragment was subcloned into the *Nhe*I/*Nco*I polylinker sites of the basic luciferase reporter plasmid, pGL3B. THP-1 and THP-1/CD14 cells were transiently transfected with the IL-12p40 promoter/luciferase reporter construct (pIL-12Pr-GL3B). Twenty-four hours posttransfection, cells were stimulated with LPS for periods of time ranging from 6 to 36 h, following which relative luciferase activity was assessed. The results show that luciferase activity could be detected by 12 h and peaked at 24 h following LPS stimulation (Fig. 5*A*). The maximum increase in luciferase activity ranged from 6- to 8-fold relative to the unstimulated cells. The cells transfected with the promoterless plasmid pGL3B did not show any increase in luciferase activity following LPS stimulation (Fig. 5*B*). Similar results were obtained for THP-1 cells, although the increase in luciferase activity was relatively lower than for the THP-1/CD14 cells transfected with pIL-12Pr-GL3B (Fig. 5*B*).

To identify the DNA sequences required for IL-12p40 transcription, we generated a series of hIL-12p40 promoter fragments (from 5′ −880 to 3′ +108 bp) by successive deletions starting from the

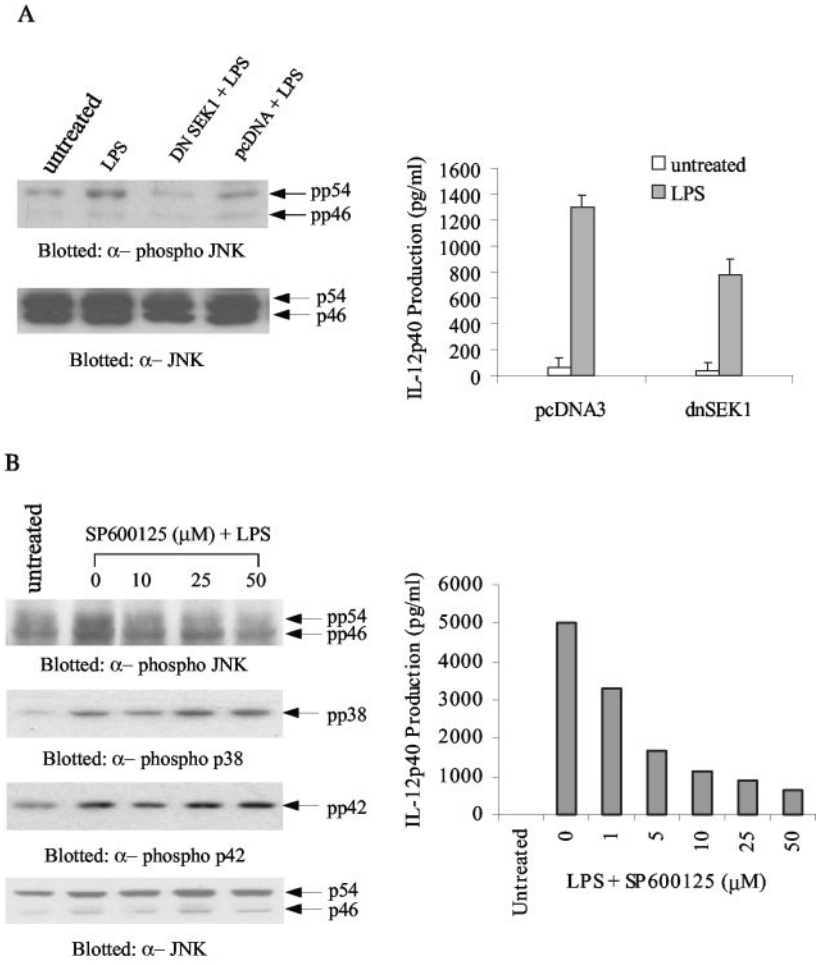

FIGURE 3. A, *Left panel*, JNK phosphorylation is inhibited by LPS stimulation in DN SEK1-transfected THP-1 cells. Cells were transfected with either a DN SEK1 kinase mutant or with control vector followed by stimulation with LPS for 10 min. Total proteins (50 μg) were subjected to SDS-PAGE analysis followed by Western blot analysis. The membranes were blotted with an anti-phospho JNK rabbit polyclonal Ab, and to control for equal loading of proteins, the membranes were stripped and reprobed with anti-JNK rabbit polyclonal Ab. *Right panel*, DN SEK1 kinase mutant inhibits LPS-induced IL-12p40 production. THP-1/CD14 cells were transfected with a DN SEK1 construct. After 12 h of transfection, cells were treated with LPS (1 μg/ml) for 48 h, following which IL-12p40 production was analyzed in the cell supernatants by ELISA. The results shown are a mean of three experiments performed. *B*, SP600125 inhibits LPS-induced IL-12p40 production in THP-1/CD14 cells. *Left panel*, THP-1/CD14 cells (1.0×10^6/ml) were treated with SP600125 at concentrations ranging from 0 to 50 μM for 2 h before LPS stimulation (1 μg/ml) for 15 min. Total proteins were analyzed for JNK phosphorylation using an anti-phospho-JNK rabbit polyclonal Ab. The same membranes were stripped and reprobed for the phosphorylation of p38 and p42/44 ERK MAPKs using the anti-phospho-p38 (pp38) or anti-phospho-p42/44 (pp42/44) Abs. To control for protein loading, the membranes were stripped and reprobed with anti-JNK rabbit polyclonal Abs. *Right panel*, Cells (0.5×10^6/ml) were treated with SP600125 at concentrations ranging from 0 to 50 μM for 2 h before stimulation with LPS followed by analysis of IL-12p40 production by ELISA. The experiment shown is representative of two different experiments.

5′ end. A number of hIL-12p40 promoter fragments were amplified, sequenced, and inserted into the luciferase expression plasmid (pGL3B). The exact size of the amplified product and the location of consensus sequences for different transcription factors identified within the IL-12p40 promoter are depicted in Figs. 4 and 6. Transfection of THP-1/CD14 cells with plasmids containing various deletions of the IL-12p40 promoter revealed that deletion of sequences from −880 to −120 bp did not affect luciferase activity compared with the cells transfected with the entire promoter sequence. However, deletion of sequences upstream of −84 bp abrogated luciferase activity (Fig. 6). Similar results were obtained for THP-1 cells (data not shown). These results suggest that DNA sequences located between −120 and −84 bp are necessary for IL-12p40 gene transcription in LPS-stimulated THP-1/CD14 cells.

-880 CCACCACGAGAGATATC T TTATTCCGCT AT TCCT GT GCATCT GCACG GA GCCCCTAGG GCCATAGATTTG

-810 TG TGCAAATGAAAT GAGGATGTAGTCTGGGTGCCCAAGGGGGGGT GCCTT GAGT GT GGTT GTCTGTATGC

IRF -730/-719

-740 CT CCCT GAGGG TATTTCACTTTC T GCTCCCATCCG CCCCTAT GAGCGAGTACCTATGAGCACAGGATGTG

-670 CACATATTT GAGTCTTATTAGT GGTACACGCAGTTTTATCATCTCCCCAG GTCT GTGTCT GTAT GAAATGTG

-600 CAT GGGT GTGT GT GT GCACGCGT GT GT TCCCACTCGGGG AAT GT GGGGAGAG GTGCATGGAGCCAAGA

NF/IL-6 -512/-505

-530 TGGGT GGTAAATAGTATGT TTCTGAAA TTAAA GGACTAAT GTGGAGGAAGGCGCCCCAGATGTACTAAAC

-460 CCTTT GCC TTCATCTCATCCTCTCTGACTT GGG AAG AACCAGGATTTT GTTTTTAAGCCCTTGGGCATACAG

SP-1 -353/-347

-390 TT GT TCCATCCC GACAT GA ACTCAGCCTCCC GTCT GA CCGCCC C T TG G CCT TCCT TCTTC CT CGATCT

-320 GT GGAACCCAGGG AATCTGCCTAGT GCT GTCTCCAA GCACCTT GGCCATGATG TAAACCCAGAGAAATTA

AP-1 -232/-226 Ets-2 -211/-206

-250 GCATCTCCATCTCCT TCC TTATTCC CCACCC AAAAGTCA TTTCCT CT TAG TCAT T ACCT GGG ATTTT GA

PU.1 -128/-123 NF-kB

-180 TGTCTAT GTT CCCT CCTCGTTATTGATACACACACAGAGAGAGACAAACAAAA AAGGAA CTTCTT GAAAT

-116/-106 C/EBP -80/-72

-110 TCCC CC AGAAGGT TTT GAGAGTT GTTTTCA ATGTTGCAA CAAGTCAGTTTCTAGT T TAAGT T TCCATCAG

TATA box Transcription Start

-40 AAAGGAGTAGAG TATATAA GTTCCAGTACCAGCAACAGCA GCAGAAGAAACAACATCTGTTTCAGGGCCA

exon 1

+30 TTGGACTCTCCGTCCTGCCCAGAGCAAGGTAAGCA CTTCCCAAGCCCCTACC TCCCTCCCCTCCCT GTGGGC

intron 1

+100 CTGCAG

FIGURE 4. Nucleotide sequence of the first exon and 5′-flanking promoter region of the *hIL-12p40* gene (GenBank accession no. U89323.). Putative *cis*-regulatory elements are boxed. The first exon is underlined and in bold type.

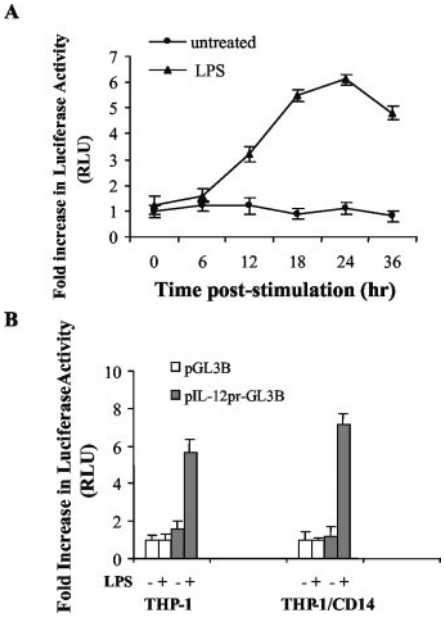

FIGURE 5. Luciferase activity in LPS-stimulated THP-1 and THP-1/CD14 cells transfected with a hIL-12p40 promoter/luciferase construct. THP-1 and THP-1/CD14 cells (1.5×10^6) were transiently cotransfected with 6 μg of either pIL-12pr-GL3B or vector control, and with 3 μg of β-galactosidase control plasmid, and allowed to grow for 24 h. *A*, THP-1 cells were treated with 1 μg/ml LPS for 6, 12, 18, 24, and 36 h. *B*, The transfected THP-1 and THP-1/CD14 cells were treated with 1 μg/ml LPS for 24 h. Following stimulation with LPS, luciferase and β-galactosidase activities were determined in the cell lysates. Cells transfected with vector pGL3B alone served as a negative control. Luciferase activity was normalized for β-galactosidase activity to give relative luciferase units (RLU). The results shown are the mean ± SD of four experiments performed in triplicate and normalized for β-galactosidase activity.

The AP-1 and NF-κB binding sites within the hIL-12p40 promoter are required for LPS-induced IL-12p40 production

IL-12p40 gene transcription has been shown to be regulated by the activation of multiple transcription factors including NF-κB, PU.1, and Ets-2 (20–27). A computer-aided analysis of the hIL-12p40 promoter sequence between −120 and −84 bp revealed the existence of consensus sequence for NF-κB (Fig. 4). To examine the role of NF-κB in LPS-induced *hIL-12p40* gene transcription, we introduced mutations in the NF-κB sequence by PCR-mediated site-directed mutagenesis (Fig. 7*A*). The fragment containing the NF-κB mutant sequence (−120 to +108 bp) was cloned into pGL3B. THP-1/CD14 cells transfected with this plasmid showed marked reduction in luciferase activity when compared with cells containing the plasmid encoding the wild-type NF-κB sequence (Fig. 7*B*). To determine the role of PU.1, we cloned IL-12p40 fragment from −131 to +108 bp in pGL3B which encodes sequences for NF-κB and PU.1 transcription factors. In this fragment, we also introduced mutations in the NF-κB sequence by PCR-mediated site directed mutagenesis followed by cloning into pGL3B (designated as IL-12p40PrNmGL3B). Transfection of THP-1/CD14 cells with IL-12p40PrNmGL3B significantly reduced the luciferase activity compared with the cells transfected with the plasmid containing wild-type NF-κB and PU.1 sequences. Furthermore, transfection of cells with the IL-12p40 promoter (−131 bp) containing mutations only in the PU.1 binding site did not reduce luciferase activity as compared with the cells transfected with the promoter containing wild-type NF-κB and PU.1 binding sites (data not shown). These results suggest that, in the promoter fragment containing sequences between −131 and −84 bp, NF-κB plays a major role in LPS-induced *IL-12p40* gene transcription.

We have demonstrated that LPS-induced IL-12p40 production is highly sensitive to DXM (Fig. 2). Because DXM has been shown to exert its biological effects by down-regulating the AP-1 transcription factor (34, 35), we hypothesized that, in addition to NF-κB, AP-1 may also play a role in LPS-induced *IL-12p40* gene transcription. To investigate the role of AP-1, we amplified a fragment spanning a distance from −232 to +108 bp and cloned it into pGL3B. This fragment encodes, in addition to AP-1, the binding sites for NF-κB, PU.1, and Ets-2 transcription factors that have been shown to be involved in *IL-12p40* gene transcription (20–27). To delineate the role of AP-1, we introduced mutations within the NF-κB, PU.1, and Ets-2 sequences in the −232- to +108-bp promoter fragment. This fragment was then inserted into pGL3B

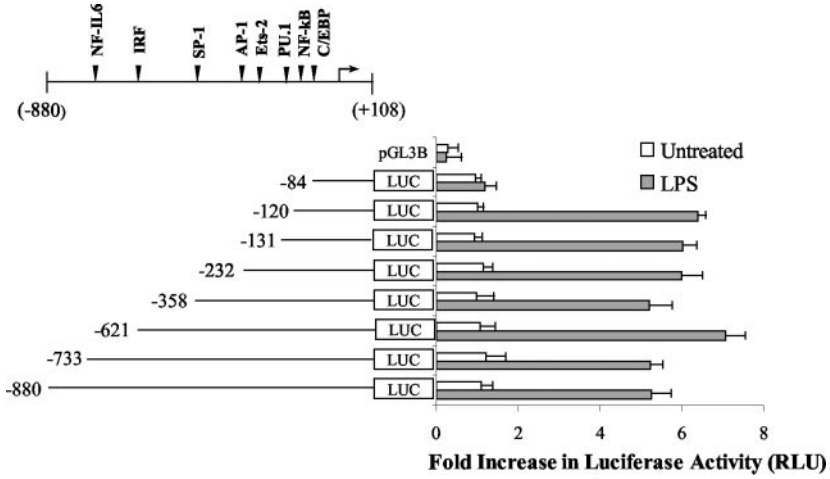

FIGURE 6. Transcriptional activities of deletion mutants of IL-12p40 promoter in LPS-stimulated THP-1/CD14 cells. *Top*, The line diagram summarizes the position of potential regulatory elements relative to the structure of eight deletion constructs used in the experiment. The putative binding sites for the transcription factors IFN regulatory factor (IRF), NF/IL-6, sp-1, AP-1, Ets-2, PU.1, NF-κB, and C/EBP are shown. *Bottom*, THP-1/CD14 cells were cotransfected with 10 μg of either IL-12p40 promoter deletion mutant construct or vector control, and with 5 μg of β-galactosidase control plasmid. After 24 h, cells were stimulated with LPS (1 μg/ml) for another 24 h. Cell lysates from unstimulated and LPS-stimulated cells were assayed for luciferase and β-galactosidase activities. Luciferase activity was normalized for β-galactosidase activity to give relative luciferase units (RLU). The results shown are the mean ± SD of four experiments performed in triplicate.

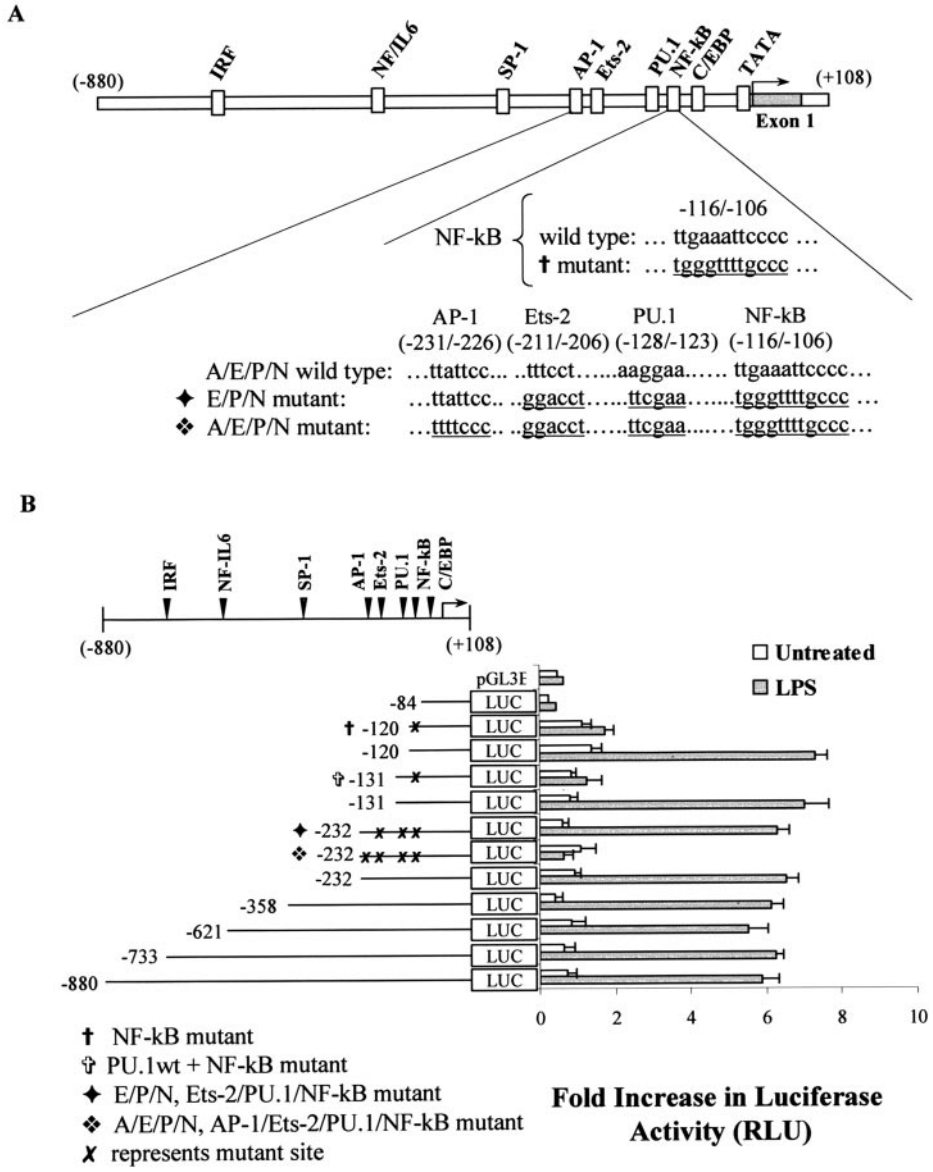

FIGURE 7. The NF-κB and AP-1 binding site within the IL-12p40 promoter is required for LPS-induced IL-12p40 production. *A*, Line diagram depicts wild-type sequences and the mutations introduced in the binding sites of transcription factors NF-κB, PU.1, Ets-2, and AP-1 in the IL-12p40 promoter fragment. The IL-12p40 fragment (−120 to +108 bp) with a mutation in the NF-κB site is designated as NF-κB mutant; the IL-12p40 fragment (−131 to +108 bp) with a mutation in the NF-κB site is designated as PU.1wt + NF-κB mutant; the IL-12p40 fragment (−232 to +108 bp) with mutations in the AP-1, Ets-2, PU.1, and NF-κB sites is designated as A/E/P/N mutant; and the IL-12p40 fragment (−232 to +108 bp) with mutations in the Ets-2, PU.1, and NF-κB sites is designated as E/P/N mutant. *B*, THP-1/CD14 cells were cotransfected with either 10 μg of wild-type or mutant constructs A/E/P/N mutant, E/P/N mutant, PU.1wt + NF-κB mutant, and NF-κB mutant, and with 5 μg of β-galactosidase control vector. The transfected cells were stimulated with LPS (1 μg/ml) for 24 h. Luciferase activity following normalization of β-galactosidase activity is shown as the mean ± SD of three experiments performed in triplicate.

(pIL-12Pr-E/P/Nm-GL3B) and used for transfection in THP-1/CD14 cells (Fig. 7*A*). To our surprise, transfection of THP-1/CD14 cells with pIL-12Pr-E/P/Nm-GL3B exhibited luciferase activity comparable with the cells transfected with the entire plasmid (Fig. 7*B*). These results of the mutational analysis of the promoter region between −232 and +108 bp suggest the presence of DNA sequences other than those of NF-κB, PU.1, and Ets-2 that are required for transcription of the *IL-12p40* gene. Because this promoter fragment also encodes a binding site for AP-1, we investigated a role for AP-1 in LPS-induced *IL-12p40* gene transcription. For this, we introduced a mutation in the consensus sequence of the AP-1 binding site, in addition to the mutations introduced earlier for the NF-κB, PU.1, and Ets-2 binding sites. This fragment was cloned into pGL3B and designated as pIL-12Pr-A/E/P/Nm-GL3B. Transfection of THP-1/CD14 cells with pIL-12Pr-A/E/P/Nm-GL3B abrogated the luciferase activity compared with the plasmid containing the wild-type AP-1, PU.1, Ets-2, and NF-κB sequences (Fig. 7*B*). However, transfection of cells with IL-12p40 promoter (−232 bp) containing mutations only in the AP-1 binding site did not significantly reduce luciferase activity compared with the cells transfected with the promoter containing wild-type NF-κB, PU.1, and Ets-2 binding sites (data not shown). These results suggested that AP-1, in addition to NF-κB, may play a role in *IL-12p40* gene transcription in LPS-stimulated human monocytic cells.

*Antisense c-*jun *and c-*fos *(AP-1) oligonucleotides inhibit IL-12p40 production in LPS-stimulated THP-1/CD14 cells*

To confirm the role of AP-1 in IL-12p40 expression, we designed antisense oligonucleotides for c-*jun* and c-*fos*, the AP-1 components, along with the control oligonucleotide containing equal number of base pairs. Cells were treated with antisense oligonucleotides for 4 h before stimulation with LPS (0.1 μg/ml in experiments 1 and 2, and 0.5 μg/ml in experiment 3) for 48 h. The results revealed that antisense c-*jun* and c-*fos* oligonucleotides significantly reduced IL-12p40 production. The c-*fos* and c-*jun* antisense oligonucleotides seem to selectively inhibit IL-12p40 production, because IL-10 production (Fig. 8) and expression of CD44, CD80, or CD86 were not affected (data not shown).

DXM and JNK inhibitor SP600125 down-regulate IL-12p40 expression by inhibiting AP-1 and NF-κB activity

To investigate whether LPS-induced IL-12p40 expression is regulated by AP-1 through JNK activation, we used DXM, and SP600125, a specific inhibitor of JNK MAPK. THP-1/CD14 cells

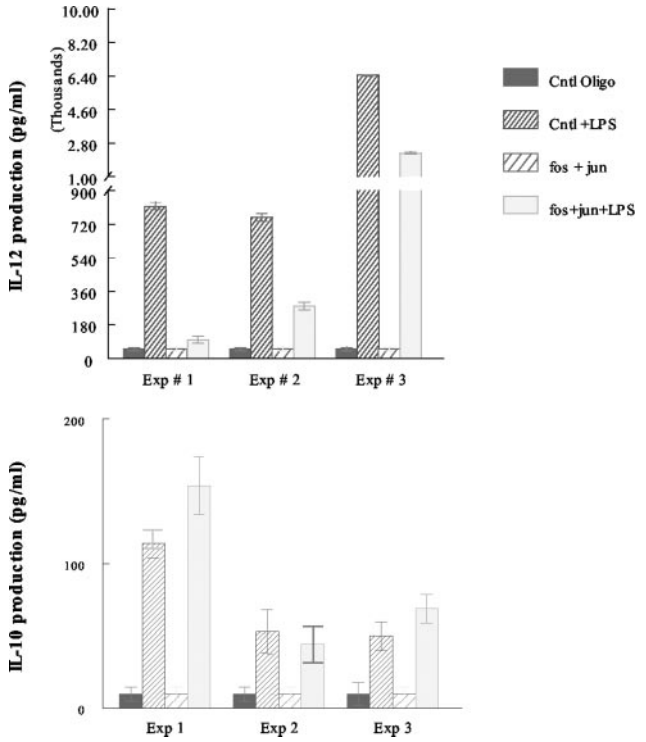

FIGURE 8. Antisense c-*jun* and c-*fos* oligonucleotides inhibit LPS-induced IL-12p40 production in THP-1/CD14 cells. Cells were treated with the c-*jun* and c-*fos* antisense oligonucleotides (10 μg/ml) for 4 h followed by stimulation with LPS (0.1 μg/ml for experiments 1 and 2, and 0.5 μg/ml for experiment 3) for 48 h. The supernatants were analyzed for IL-12p40 and IL-10 production.

were transfected with pGL3B containing a series of successive 5′ deletions derived from −880 to +108 bp of the hIL-12p40 promoter as described above. The transfected cells were cultured for 2 h in the presence or the absence of either DXM or SP600125. As a control, we treated transfected cells with PD98059, the ERK MAPK inhibitor, before stimulation with LPS. Luciferase activity was measured after 24 h.

As observed above, transfection of THP-1/CD14 cells with plasmids containing deletion of sequences spanning −880 to −131 bp from the hIL-12 p40 promoter region revealed a 6- to 8-fold increase in the luciferase activity in LPS-stimulated cells, compared with the unstimulated cells or cells transfected with the control plasmid (Fig. 9*A*). Pretreatment of same cells with DXM or SP600125 abrogated the luciferase activity (Fig. 9*A*). Similar results were observed in cells transfected with a plasmid (pIL-12Pr-E/P/Nm-GL3B) containing IL-12p40 promoter sequences (−232 to +108 bp) showing mutations in the binding sites for NF-κB, PU.1, and Ets-2. Both DXM and SP600125 inhibited the luciferase activity in THP-1/CD14 cells transfected with pIL-12Pr-E/P/Nm-GL3B following LPS stimulation (Fig. 9*A*). As observed above, transfection of THP-1/CD14 cells with the plasmid, pIL-12-A/E/P/Nm-GL3B, containing mutations in the consensus sequences for AP-1, Ets-2, PU.1, and NF-κB binding sites, did not increase the luciferase activity upon LPS stimulation. Our deletion analyses also demonstrated the sensitivity of NF-κB activation to DXM. Cells transfected with the constructs spanning from −131 to +108 bp that included a NF-κB but not an AP-1 sequence, exhibited luciferase activity following LPS stimulation, and this activity was lost upon prior treatment with DXM or SP600125 (Fig. 9*A*). Under the same experimental conditions, pretreatment of cells with PD98059 did not have any effect (Fig. 9*A*). To confirm that PD98059 can inhibit the luciferase activity of the reporter gene linked to the promoter of an ERK-responsive gene in our THP-1/

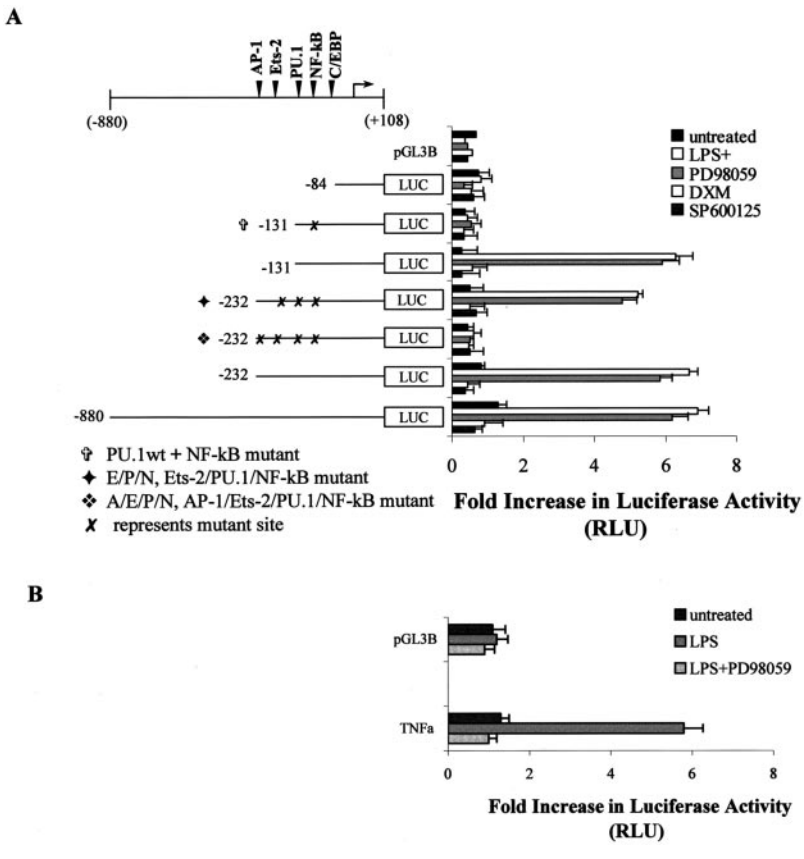

FIGURE 9. *A*, DXM and SP600125 inhibit IL-12p40 expression by inhibiting AP-1 and NF-κB activity. THP-1/CD14 cells (1.5×10^6) were cotransfected with either 10 μg of wild-type or mutant constructs A/E/P/N mutant, E/P/N mutant, and PU.1wt + NF-κB mutant, and with 5 μg of β-galactosidase control vector. The transfected cells were pretreated with either 25 nM DXM, 25 μM SP600125, or 15 μM PD98059 as a control for 2 h followed by stimulation with 1 μg/ml LPS for 24 h. Unstimulated, LPS-stimulated (LPS), LPS plus DXM (DXM)-, LPS plus SP600125 (SP600125)-, or LPS plus PD98059 (PD98059)-treated cells were harvested, and their lysates were assessed for luciferase and β-galactosidase activities. The results shown are the mean ± SD of three experiments performed in triplicate and normalized by β-galactosidase activity. *B*, PD98059 can inhibit an ERK-responsive gene, *TNF-α*, in THP-1/CD14 cells. THP-1/CD14 cells transfected with the TNF-α promoter linked to the luciferase reporter gene were stimulated with LPS for 24 h followed by the assessment of luciferase activity as described above. The results shown are the mean ± SD of two experiments performed in triplicate following normalization for β-galactosidase activity.

CD14 cell system, we transfected cells with TNF-α promoter linked to the luciferase reporter gene (kindly provided by Dr. D. Wilkinson (Ottawa Health Research Institute, Ottawa, Ontario, Canada). We have previously shown that PD98059 inhibited LPS-induced TNF-α production in THP-1 cells in a dose-dependent manner (48). In this study, we show that PD98059 inhibited LPS-induced luciferase activity in cells transfected with the plasmid containing TNF-α promoter compared with the cells transfected with the control plasmid (Fig. 9*B*). Taken together, these results suggest that both DXM and SP600125 inhibited IL-12p40 transcription by inhibiting AP-1 activation in addition to that of NF-κB.

DXM and SP600125 inhibit NF-κB and AP-1 binding to the IL-12p40 promoter in LPS-stimulated THP-1/CD14 cells and normal monocytes

To confirm the role of AP-1 and NF-κB in the regulation of *hIL-12p40* gene transcription, we investigated whether LPS stimulation of THP-1/CD14 cells and normal monocytes induced the binding of NF-κB and AP-1 to their respective NF-κB and AP-1-binding sites present in the hIL-12p40 promoter. THP-1/CD14 cells and normal monocytes were stimulated with LPS over a period of time ranging from 0 to 60 min, and the nuclear extracts were analyzed in a gel shift assay for binding to AP-1 and NF-κB oligonucleotide probes corresponding to the AP-1 and NF-κB binding sites, respectively, in the IL-12p40 promoter. Because it was difficult to obtain sufficient numbers of monocytes from one donor, cells were stimulated for only 30 and 45 min. The results show that significant binding of AP-1 and NF-κB to the AP-1 and NF-κB oligonucleotides, respectively, occurred 30–45 min following stimulation of THP-1/CD14 cells (Fig. 10*A*) and normal monocytes (*B*) with LPS (18). This binding was completely blocked by competition with their respective cold AP-1 and NF-κB oligonucleotides indicating their specificity (Fig. 10*A*). In contrast, mutant oligonucleotides (mNF-κB and mAP-1), unlike their counterpart unmutated oligonucleotides, failed to compete for binding of NF-κB and AP-1 transcription factors to their respective NF-κB- and AP-1-labeled oligonucleotide probes (Fig. 10*A*). The mutations in the mNF-κB and mAP-1 oligonucleotides corresponded to the mutations in the IL-12p40 promoter construct, pIL-12Pr-A/E/P/Nm-GL3B, used in the luciferase reporter assays as shown in Fig. 7. In addition, supershift analyses were performed to confirm the identity of the transcription factors NF-κB and AP-1 by using specific mouse anti-NF-κB p50 and p65 mAbs and rabbit anti-c-*fos* and rabbit anti-c-*jun* polyclonal Abs, respectively. Incubation of nuclear extracts obtained from both THP-1/CD14 cells and monocytes with oligonucleotide probes and either anti-p50 or p65 NF-κB Abs, or anti-c-*fos* or anti-c-*jun* Abs revealed bands of higher molecular mass (Fig. 10). To determine whether DXM and SP600125 inhibited binding of AP-1 and NF-κB to their binding sites in the IL-12p40 promoter, THP-1/CD14 cells and normal monocytes were treated with DXM or SP600125 for 2 h before LPS stimulation for 30 or 45 min followed by the analysis of AP-1 and NF-κB binding to their corresponding oligonucleotide probes. The results show that both DXM and SP600125 inhibited the binding of AP-1 and NF-κB transcription factors to the AP-1 and NF-κB probes, respectively, in both LPS-stimulated normal monocytes and THP-1/CD14 cells (Fig. 10). Taken together, the results suggest that *IL-12p40* gene transcription may be regulated by AP-1 in addition to that of NF-κB transcription factors through JNK activation.

Discussion

The bacterial cell wall component LPS is a potent inducer of IL-12 in monocytes (44, 45). IL-12 may be induced as a result of the association of LPS with the LPS-binding plasma protein, and con-

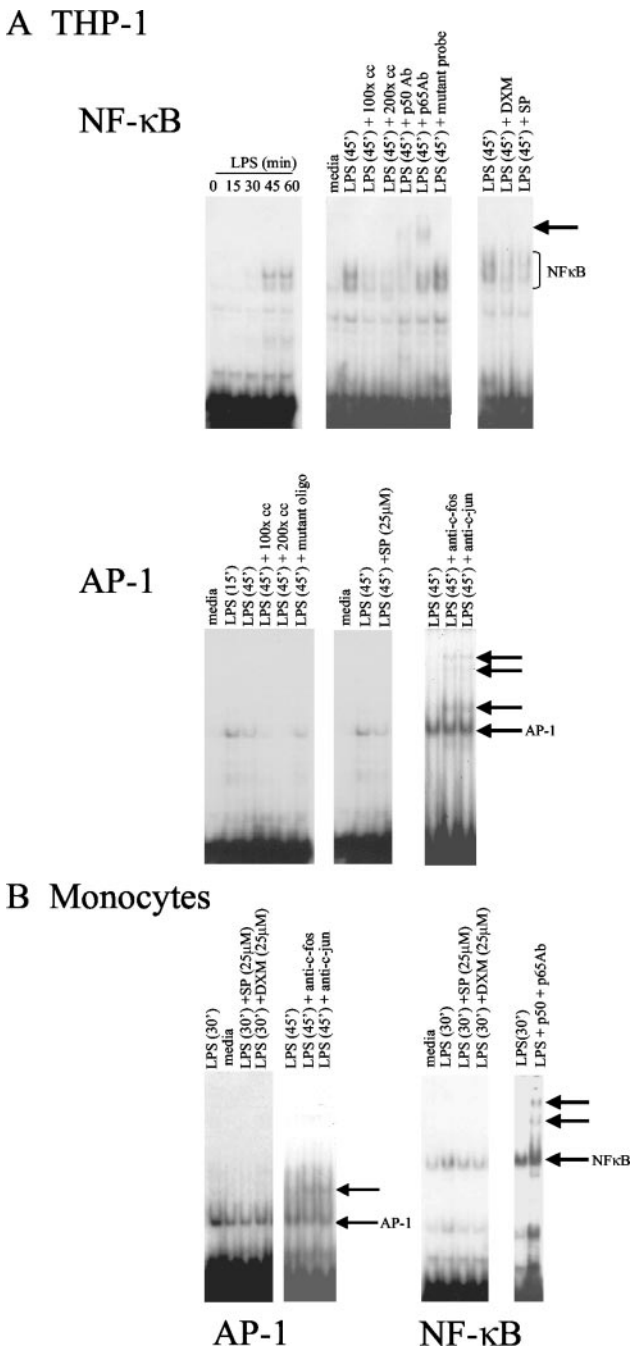

FIGURE 10. DXM and SP600125 inhibit NF-κB and AP-1 binding to the IL-12p40 promoter in LPS-stimulated THP-1/CD14 cells (*A*) and normal human monocytes (*B*). THP-1/CD14 cells and monocytes were stimulated with LPS (1 μg/ml) for times ranging from 0 to 60 min. To determine the effects of DXM and SP600125 on LPS-induced NF-κB and AP-1 activation, cells were treated with either DXM (25 nM) or SP600125 (25 μM) for 2 h before stimulation with LPS. Nuclear extracts containing 5 μg of proteins were incubated for 1 h with ^{32}P-labeled oligonucleotides corresponding to the AP-1 or NF-κB sequences derived from the IL-12p40 promoter. To determine the specificity of AP-1 and NF-κB transcription factor binding, the nuclear extracts were incubated with unlabeled wild-type or mutant oligonucleotides (100–200×) corresponding to the AP-1 and NF-κB sequences, respectively. The supershift analysis for the NF-κB transcription factor was performed by treating the nuclear extracts obtained from both THP-1/CD14 cells (*A*) and monocytes (*B*) with oligonucleotide probes and either anti-p50 or -p65 NF-κB Abs, or the isotype control Abs. Similarly, for the AP-1 transcription factor, the nuclear extracts were incubated with oligonucleotide probes and anti-c-*fos*, anti-c-*jun* polyclonal Abs, or the control Abs. The supershift bands for NF-κB and AP-1 are indicated by arrows.

sequential binding of the LPS/LPS-binding plasma protein complex with the CD14/Toll receptor complex expressed on cells of monocytic lineage (49, 50). In this study, we show that DXM, an anti-inflammatory and immunosuppressive glucocorticoid that is widely used in the treatment of inflammation and a number of autoimmune disorders (28), inhibited the synthesis of LPS-induced IL-12 production (30, 31). We investigated the molecular mechanism by which DXM inhibits IL-12p40 production and in particular the role of MAPK and the transcription factors involved in IL-12p40 regulation. We show that DXM inhibited LPS-induced IL-12p40 production, suggesting an involvement of JNK. The role of JNK was confirmed by using specific inhibitors of JNK activation, SP600125 and a DN SEK-1 kinase mutant. Extensive deletion analysis of IL-12p40 promoter sequences revealed that a DNA element encompassing the AP-1-binding site, in addition to that of NF-κB, may be involved in *IL-12p40* gene transcription. The role of AP-1 was further confirmed by using antisense c-*jun* and c-*fos* oligonucleotides, resulting in significantly reduced LPS-induced IL-12p40 production. Furthermore, we show that DXM and SP600125 interfered with *IL-12p40* gene transcription by inhibiting the activation of AP-1 in addition to that of NF-κB.

It has been shown that IL-10 produced endogenously by LPS-stimulated monocytes inhibits IL-12 production (11). To avoid negative feedback regulation by the endogenous IL-10, we used THP-1/CD14 cells that are refractory to the IL-10-mediated biological effects (42). We have previously shown that, in contrast to THP-1 cells, CD14 was constitutively expressed on THP-1/CD14 cells and exhibited enhanced LPS-mediated responses (42). Transfection of THP-1/CD14 cells with the IL-12p40 promoter linked to a luciferase gene revealed higher luciferase activity following LPS stimulation as compared with THP-1 cells transfected with the same constructs, and hence were used throughout this study.

Multiple transcription factors, including NF-κB, PU.1, and Ets-2, have been implicated in *IL-12p40* gene transcription (20–27). By cloning and mutagenesis of the IL-12p40 promoter, we provide evidence for the previously unrecognized role of AP-1 in addition to that of NF-κB in the regulation of LPS-induced IL-12p40 production. We show that mutation of NF-κB binding site in the IL-12p40 promoter constructs (−120 bp and −131 bp; pIL-12p40Pr-Nm-GL3B) reduced the luciferase activity, thereby confirming a role for NF-κB. Because the IL-12p40 promoter construct (−131bp; pIL-12p40Pr-Nm-GL3B) also contains wild-type PU.1 in addition to mutant NF-κB sequences, loss of luciferase activity following LPS stimulation suggest that PU.1 may not be involved in *IL-12p40* gene activation in our model system. However, mutagenesis of NF-κB, PU.1, and Ets-2 binding sites in the IL-12p40 promoter construct (−232 bp; pIL-12Pr-E/P/Nm-GL3B) failed to reduce luciferase activity, suggesting the presence of binding sites for other transcription factors present in the IL-12p40 promoter that may cooperate with NF-κB or may substitute for NF-κB activity. Introduction of mutations in the AP-1 binding site, in addition to those of NF-κB, PU.1, and Ets-2 (−232 bp; pIL-12Pr-A/E/P/Nm-GL3B), abrogated the luciferase activity, thereby suggesting a role for AP-1 in *IL-12p40* gene transcription. Conversely, mutagenesis of the AP-1 site alone in this IL-12p40 promoter construct failed to abrogate luciferase activity (data not shown). These results are contrary to the observations provided by Becker et al. (25) whereby mutations of NF-κB, Ets, and C/EBP sites reduced IL-12p40 promoter activity even though the AP-1 site was left intact. Although the reasons for this discrepancy are not clear, it appears that *IL-12p40* gene transcription may be subject to differential regulation depending on the cell type and the nature of the external stimuli used. In contrast to our studies, Becker et al. (25) have analyzed the IL-12p40 promoter by using murine RAW264 macrophage cells stimulated with LPS and IFN-γ. It is likely that stimulation of cells with IFN-γ in addition to LPS may activate a distinct set of transcription factors required for *IL-12p40* gene activation, thereby revealing complex mechanisms involved in *IL-12p40* gene regulation.

The role of AP-1 in IL-12p40 regulation was confirmed by an alternative approach using antisense oligonucleotides specific for c-*jun* and c-*fos*, which significantly inhibited IL-12p40 production in LPS-stimulated THP-1/CD14 cells. This inhibition was specific, because these oligonucleotides failed to inhibit IL-10 production under the same experimental conditions. While this work was in progress, Zhu et al. (26) demonstrated the involvement of an AP-1 sequence in murine IL-12p40 expression using RAW264.7. Although the human and murine IL-12p40 promoter sequences are distinct, results suggest that the AP-1 transcription factor may play a critical role in both human and murine *IL-12p40* gene transcription.

AP-1 has been shown to play a key role in the expression of a number of cytokines (51–53). AP-1 is a heterodimeric transcription factor comprised of members of the *jun* (c-Jun, JunB, and JunD) and *fos* (c-*fos*, Fra-1, Dra-2, FosB, and FosB2) proto-oncogene families (54). Members of the *fos* and *jun* families have been shown to dimerize via their leucine zipper domain with a variety of transcription factors including CREB/activating transcription factor, Maf, NFAT, and glucocorticoid receptor (54–56). Various Fos and Jun proteins interact with the promoters of cytokine genes either individually as AP-1 dimers, or in cooperation with other transcription factors such as NF-κB, NFAT, CREB/activating transcription factor, etc. (54, 57). For example, the regulation of IL-4, IL-5, and GM-CSF requires the formation of NFAT/AP-1 complexes in T cells (51–53). Similarly, in LPS-stimulated THP-1 cells, c-Jun-containing complexes have been shown to interact with NF-κB proteins p50/p65, and synergistically enhance the TNF-α promoter activity (58). The results of this study reveal the involvement of NF-κB and AP-1 in IL-12p40 regulation. It is not clear whether both of these transcription factors can cooperate in *IL-12p40* gene transcription when an intact IL-12p40 promoter is provided. It is likely that the level of this putative cooperation is modulated by interaction with other cellular signaling molecules activated by different external stimuli depending on the cell type. Further studies are needed to understand the interaction of NF-κB and AP-1 and possibly other transcription factors necessary for IL-12p40 regulation.

We have also investigated the upstream signaling events that lead to AP-1 and NF-κB activation resulting in IL-12p40 production. We primarily studied the role of MAPK in AP-1 and NF-κB activation and in IL-12p40 production. Recently, p38 MAPK has been shown to regulate IL-12p40 production in dendritic cells stimulated by TNF-α, and anti-CD40 Abs (59, 60) as well as in IFN-γ-primed human monocytic cells (61). Similarly, p38 has been shown to regulate IL-12p40 induction in murine macrophages and in a p38 MAPK knockout mouse model (45, 62). Contrary to these observations, IL-12p40 production in LPS- and IFN-γ-induced human monocytes has been shown to be independent of p38 activation (45, 63). In this study, we show that p38 MAPK did not regulate IL-12p40 production in LPS-stimulated human monocytes. Conflicting results on the role of p38 MAPK may be due to the negative feedback regulation of IL-12 by immunoregulatory cytokines such as IL-10, as well as differences in cell types and the stimulus used to activate distinct signaling pathways.

The role of JNK in the regulation of IL-12p40 production was initially studied by DXM, which mediates its effects by interfering with JNK phosphorylation and AP-1 activation (32, 33). We provide evidence for the role of JNK MAPK in the regulation of

LPS-induced IL-12p40 production, by using the specific JNK inhibitor, SP600125, and by DN mutant of MKK4/SEK1 kinase. MKK4 is an essential component of the JNK signal transduction pathway, and disruption of the *MKK4* gene specifically blocks JNK activity (46, 47, 64, 65). The data presented in this report also suggest the involvement of JNK in IL-12p40 production through the activation of AP-1 and NF-κB transcription factors. Our results show that both DXM, and SP600125 significantly decreased the luciferase activity in THP-1/CD14 cells transfected with IL-12p40 promoter constructs containing mutations in the binding sites for NF-κB (−120 and −131 bp; pIL-12p40Pr-Nm-GL3B), and NF-κB, PU.1, and Ets-2 transcription factors (−232 bp; pIL-12Pr-E/P/Nm-GL3B). In addition, by using mobility gel shift assays, we show that both DXM and SP600125 inhibited the binding of AP-1 and NF-κB to their respective oligonucleotides in LPS-stimulated THP-1/CD14 cells. Taken together, our results suggest for the first time a role for JNK in hIL-12p40 production via the activation of AP-1. Our results also suggest the involvement of JNK in the NF-κB pathway leading to IL-12p40 production. Although it is not known whether JNK directly interact with the members of the NF-κB transcription factors, JNK has been shown to influence the NF-κB pathway by regulating IκBα activation (57). Furthermore, c-Jun-containing complexes have been shown to interact with NF-κB proteins p50/p65 (66, 67). Keeping in view these observations, our results may suggest a potential cooperation between AP-1 and NF-κB pathways in the regulation of IL-12p40 production in human monocytic cells.

The glucocorticoids regulate many biological processes through their intracellular glucocorticoid receptors. Following binding to the glucocorticoids, the glucocorticoid receptors homodimerize and interact with specific DNA sequences termed glucocorticoid response elements (GREs) for transcription of glucocorticoid-responsive genes (68). Initially, the glucocorticoids were thought to mediate their therapeutic effects through the transcription of glucocorticoid-responsive genes. Recently, the glucocorticoids have been shown to repress transcription by inhibiting the activity of a number of transcription factors including AP-1 and NF-κB independent of binding through the GREs (69, 70). Whether DXM inhibits IL-12p40 production by trans-repressing AP-1 and NF-κB activity independent of GREs needs investigation.

The unique role of IL-12 in the development of cell-mediated immunity explains its key role in the pathogenesis of a number of inflammatory and autoimmune disorders such as multiple sclerosis and experimental autoimmune encephalomyelitis. Inhibiting the action of IL-12 by agents such as DXM has been shown to block the development and progression of these disorders. In summary, the results of this study show for the first time that DXM is a potent inhibitor of IL-12p40 production by LPS-stimulated monocytic cells, and DXM exerts its inhibitory effect on LPS-induced IL-12p40 production by inhibiting the activation of JNK MAPK and AP-1 transcription factor in addition to that of NF-κB. Our findings of the role of AP-1 and JNK MAPK in the induction of hIL-12p40 by LPS-stimulated monocytic cells, raise the possibility of the development of novel active analogs of DXM with more efficient inhibition of IL-12 production that may provide new therapeutic tools for the treatment of inflammation and autoimmune diseases.

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