Interleukin-10 (IL-10), a pleiotropic cytokine that inhibits inflammatory and cell-mediated immune responses, is produced by a wide variety of cell types including T and B cells and monocytes/macrophages. Regulation of pro- and anti-inflammatory cytokines has been suggested to involve distinct signaling pathways. In this study, we investigated the regulation of the human IL-10 (hIL-10) promoter in the human monocytic cell line THP-1 following activation with lipopolysaccharide (LPS). Analysis of hIL-10 promoter sequences revealed that DNA sequences located between base pairs −652 and −571 are necessary for IL-10 transcription. A computer analysis of the promoter sequence between base pairs −652 and −571 revealed the existence of consensus sequences for Sp1, PEA1, YY1, and Epstein-Barr virus-specific nuclear antigen-2 (EBNA-2)-like transcription factors. THP-1 cells transfected with a plasmid containing mutant Sp1 abrogated the promoter activity, whereas plasmids containing the sequences for PEA1, YY1, and EBNA-2-like transcription factors did not influence hIL-10 promoter activity. To understand the events upstream of Sp1 activation, we investigated the role of p38 and extracellular signal-regulated kinase mitogen-activated protein kinases by using their specific inhibitors. SB202190 and SB203580, the p38-specific inhibitors, inhibited LPS-induced IL-10 production. In contrast, PD98059, a specific inhibitor of extracellular signal-regulated kinase kinases, failed to modulate IL-10 production. Furthermore, SB203580 inhibited LPS-induced activation of Sp1, as well as the promoter activity in cells transfected with a plasmid containing the Sp1 consensus sequence. These results suggest that p38 mitogen-activated protein kinase regulates LPS-induced activation of Sp1, which in turn regulates transcription of the hIL-10 gene.

An appropriate balance between pro- and anti-inflammatory influences in the immune response is critical in the resolution of many pathological conditions. Interleukin-10 (IL-10), 1 a cytokine that inhibits inflammatory and cell-mediated immune responses (1), has enormous potential for the treatment of inflammatory and autoimmune disorders. Human IL-10 (hIL-10), a nonglycosylated 178-amino acid polypeptide, is encoded by a gene located on chromosome 1q and has more than 73% amino acid sequence homology with murine IL-10 (mIL-10) (1–3). IL-10 is a pleiotropic molecule that is produced by a wide variety of cell types, including CD4+ Th0 and Th2 cells, CD8+ T cells (4), B cells (1, 5, 6), and monocytes/macrophages (7). The major biological effects of IL-10 include inhibition of antigen-presenting cell-dependent cytokine synthesis by Th1 cells, costimulation of mast cell growth, and costimulation of thymocyte growth in the presence of IL-2 and/or IL-4 (1, 8). IL-10 inhibits antigen-driven activity of both Th1 and Th2 subsets (1, 4, 8), although it facilitates the induction of Th2 cell types. IL-10 exhibits stimulatory effects on B cell growth and differentiation (6, 9, 10) and acts as an autocrine growth factor for Ly-1+ B cells, which are important in murine models of autoimmune disease (11).

The potent action of IL-10 on macrophages, particularly at the level of monokine production (1, 7, 8), supports an important role for IL-10 not only in the regulation of T cell responses (1, 4, 8) but also in acute inflammatory and autoimmune responses (12–14). In mice, IL-10 administration has been shown to inhibit a number of immunological effects, including delayed type hypersensitivity, alterations in vascular permeability, and increases in footpad cytokine production (15). Conversely, IL-10 transgenic mice were shown to be unable to limit the growth of immunogenic tumor cells (16). In contrast, IL-10 knockout mice demonstrate a state of chronic inflammation (12), severe disease in experimental allergic encephalomyelitis (13), and bronchopulmonary aspergillosis (14), suggesting that IL-10 plays a beneficial role in controlling the harmful inflammatory response in these conditions. In humans, high levels of IL-10 have been shown to be produced in patients with HIV infection (17–19) and in septic shock (20). There is also evidence to suggest that polymorphism in the hIL-10 promoter

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1 The abbreviations used are: IL, interleukin; hIL, human interleukin; mL, murine interleukin; EBNA-2, Epstein-Barr virus-specific nuclear antigen-2; ERK, extracellular signal-regulated kinase; JNK, c-Jun N terminal kinase; LPS, lipopolysaccharide; MAP, mitogen-activated protein; MAPK, MAP kinase; TNF, tumor necrosis factor; bp, base pairs; PCR, polymerase chain reaction; TLR, Toll-like receptor(s).
region is associated with altered IL-10 expression in autoimmune diseases including multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus (21).

The molecular mechanisms underlying the regulation of cytokine synthesis in mononuclear phagocytes are not fully known. LPS is perhaps the best characterized monomeric mitogen, which, following interaction with its receptor CD14, induces first proinflammatory (IL-1, TNF-α, etc.) and then anti-inflammatory (IL-10, sTNF-R, and IL-1R antagonist) cytokines (7, 22). LPS-induced cell signaling is known to activate protein tyrosine kinases (23) and the MAP kinases p38, p44/42 extracellular signal-regulated kinase (ERK), and p54 (stress-activated protein kinase/c-Jun N-terminal kinase (JNK)) (24–27). A recent report has indicated that IL-10 production is dependent on protein tyrosine kinases and protein kinase C activation in a murine cell line (28). In addition, factors that elevate cAMP have been suggested to be involved in the regulation of monocytic IL-10 synthesis, primarily at the mRNA level (29, 30). Recently, it has been suggested that p38 MAPK is involved in the regulation of IL-10 production (31).

Regulation of gene expression for several pro- and anti-inflammatory cytokines has been studied. Transcription factors including Rel, C/EBP, AP-1, and NF-κB have been implicated in the regulation of proinflammatory cytokine genes (32–37). However, very little is known about the regulation of the hIL-10 gene and the involvement of MAP kinases in this process. To gain insight into hIL-10 gene regulation, we have employed promonocytic THP-1 cells that produce IL-10, IL-12, and TNF-α following LPS stimulation as do normal human monocytes. Using mutagenesis, we analyzed the promoter sequence of the hIL-10 gene, and we present evidence that an element located at −650 bp, encompassing a Sp1 consensus sequence is involved in the transcription of the hIL-10 gene. This was further demonstrated by introducing a mutation in the Sp1 consensus sequence that abrogated IL-10 promoter activity. Furthermore, the Sp1 transcription factor is induced by LPS stimulation and is selectively regulated by the p38 MAP kinase.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Cell Culture, and Reagents—**THP-1, a promonocytic cell line, was obtained from the American Type Culture Collection (ATCC; Manassas, VA). 5–15% of these cells express CD14 on their surface. THP-1 cells transfected with a plasmid containing CD14 cDNA sequence (THP-1/CD14) were kindly provided by Dr. Richard Ulrich (The Scripps Research Institute, La Jolla, CA) (Fig. 1). Cells were cultured in Iscove’s modified Dulbecco’s medium (Sigma) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, 10 μM HEPES, and 2 μM glutamine. PD98059 (Calbiochem), an inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase-1 kinase, selectively blocks the activity of ERK MAP kinase and has no effect on the activity of other serine threonine protein kinases including Raf-1, p38, and JNK MAP kinases, protein kinase C, and protein kinase A (24, 38). The pyridinyl imidazole SB202190 and SB203580 (Calbiochem), potent inhibitors of p38 and p38β MAP kinases, have no significant effect on the activity of the ERK or JNK MAP kinase subgroups (24, 39). LPS was purchased from Sigma.

**Cell Stimulation, Collection of Culture Supernatants, and Measurement of IL-10 by ELISA—**THP-1 cells were cultured at concentrations of 0.5 × 10⁶ cells/ml in 24-well culture plates (Falcon, Becton-Dickinson, Franklin Lakes, NJ). Cells were left unstimulated or were treated for 48 h with several agents for different periods of time, following which they were stimulated with 1 μg/ml LPS. The supernatants were frozen at −70 °C and thawed at the time of analysis. IL-10 levels were measured by enzyme-linked immunosorbent assay by using two different monoclonal antibodies that recognize distinct epitopes, as described (17, 40).

**RNA Isolation and Semi-quantitative Reverse Transcriptase-based Polymerase Chain Reaction (PCR) for IL-10—**Total RNA was extracted as described (40) using a monophase solution containing guanidine isothiocyanate and phenol (Tri Reagent solution; Molecular Research Center, Inc., Cincinnati, OH). Total RNA (1 μg) was reverse transcribed by using Moloney murine leukemia virus reverse transcriptase (PerkinElmer Life Sciences). Equal aliquots (5 μl) of cDNA equivalent to 100 ng of RNA were subsequently amplified for IL-10 and β-actin.

The oligonucleotide primer sequences for IL-10 and β-actin (hIL-10 gene, LPS dose (29 g/ml LPS). The supernatants were frozen at −70 °C and thawed at the time of analysis. IL-10 levels were measured by enzyme-linked immunosorbent assay by using two different monoclonal antibodies that recognize distinct epitopes, as described (17, 40). Following incubation, cells were stimulated with 1 μg/ml LPS and were cultured for another 24 h. Cells were harvested and then assayed for luciferase and β-galactosidase activity by using a luciferase assay kit and β-galactosidase assay kit purchased from Promega in a BioOrbit 1250 Luminometer (Fischer).

**Immunoprecipitation and Western Blot Analysis—**Cells were subjected to flow cytometric analysis as described (17, 40). Briefly, cells were stained with 3 μl of fluorescein isothiocyanate-labeled anti-CD14 monoclonal antibodies (Becton Dickinson) along with isotype (IgG2b)-matched control antibodies (Becton Dickinson). The gates were set in accordance with gates obtained with the isotype-matched control antibodies. Population data were acquired on a Becton Dickinson FACScal flow cytometer, and figures were generated using the WinMDI software package (J. Trotter, Scripps Institute, San Diego, CA).

**Construction of Luciferase Reporter Gene Vectors—**A series of hIL-10 promoter fragments (see Fig. 3; fragment −890 to +120; GenBank™ accession number X78437) were amplified from genomic DNA by PCR. The primers with restriction sites used to amplify the hIL-10 promoter fragments from genomic DNA are shown in Table 1. The amplification consisted of denaturation at 95 °C for 2 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 1 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 PCRII-TOPO vector, and the sequences were confirmed. The correct insertions were subcloned into the XbaI/EcoRI restriction site of pGL3 basic luciferase plasmid, and sequences were confirmed again. All DNA sequencing was performed by the Biotechnology Research Institute (University of Ottawa). A site-directed mutation of the Sp1-binding sequence (ccgccc) was generated by PCR using mutagenic primers (Table I) to substitute cytosine with guanine at −651 and cytosine with adenine at −638 (see Fig. 6A). The fragment containing the Sp1 mutation (−650 to +120 bp) was inserted into the pGL3B reporter vector.

**Transient Transfection of Cells and Measurement of Luciferase Activity—**Transfection of THP-1 and CD14-transfected THP-1 (THP-1/CD14) cells with plasmids containing various IL-10 promoter fragments was performed using LipofectAmine Reagent (Life Technologies, Inc.) following the manufacturer’s instructions. 10 μg of the test plasmid and 5 μg of pSV-β-galactosidase internal control vector (Promega) were incubated for 45 min with 10 μl of LipofectAmine reagent in 200 μl of OPTI-MEM I Reduced Serum Medium (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 stimulated with 1 μg/ml LPS and were cultured for another 24 h. Cells were harvested and then assayed for luciferase and β-galactosidase activity by using a luciferase assay kit and β-galactosidase assay kit purchased from Promega in a BioOrbit 1250 Luminometer (Fischer).

**Regulation of hIL-10 Promoter by Sp1 and p38 MAPK**
were precleared with protein A-Sepharose 4B beads (Amersham Pharmacia Biotech) for 1 h at 4°C followed by incubation for 2 h at 4°C with protein A-Sepharose beads and antibodies as indicated in the figure legends. Anti-p38 and anti-p42/44 rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Immune complexes were washed three times with the lysis buffer, boiled for 5 min in SDS-polyacrylamide gel electrophoresis buffer, and subjected to electrophoresis on 8% polyacrylamide SDS gels. Proteins were transferred to Immobilon™-P membranes (Millipore Corp., Bedford, MA), and the membranes were probed for phosphorylated p38 and p42/44 proteins using anti-phosphotyrosine 4G10 (UBL) antibodies. The immunoblots were developed by ECL (Amersham Pharmacia Biotech) as per the manufacturer's instructions.

Statistical Analysis—All transfection studies were performed in triplicate dishes in 3–5 separate experiments. The results are expressed as mean ± S.D.

### RESULTS

**THP-1 Cells Produce IL-10 in Response to LPS**—To understand LPS-induced IL-10 regulation in human monocytes, we employed two promonocytic cell lines, THP-1 and THP-1/CD14. CD14 was found to be expressed on 10–15% of THP-1 cells, and this number increased to more than 50% after stimulation with LPS (Fig. 1). Since production of cytokines by LPS-stimulated monocytic cell lines is critically dependent on the level of CD14 expression, we employed THP-1 cells transfected with CD14 (THP-1/CD14) for analysis of the regulation of IL-10 production. All THP-1/CD14 cells constitutively expressed very high levels of CD14 on their surfaces compared with untransfected cells (15%) (Fig. 1). Stimulation of THP-1/CD14 cells...
Regulation of hIL-10 Promoter by Sp1 and p38 MAPK

The IL-10-promoter/luciferase reporter construct (pIL-10Pr-GL3B). After 24 h of transfection, the cells were stimulated with LPS for varying 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 stimulation with LPS (Fig. 4A). 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 stimulation with LPS (Fig. 4B). 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 the pGL3B containing the IL-10 promoter (Fig. 4B).

To determine the DNA sequences in the hIL-10 promoter that are required for IL-10 transcription, a series of hIL-10 promoter fragments (from 5′ - 890 to 3′ + 120 bp relative to the +1 transcription site of the hIL-10 gene) were produced by generating successive deletions starting from the 5′-end. Various hIL-10 promoter fragments were amplified from the hIL-10 promoter region, sequenced, and inserted into the luciferase expression plasmid (pGL3B). The exact size of the amplified product and the location of consensus sequences for various transcription factors identified within the hIL-10 promoter (Fig. 3) are depicted in Fig. 5 (left panel). Examination of the DNA sequences within the hIL-10 promoter region containing various deletions revealed that deletion of sequences from −890 to −652 bp had no effect on luciferase activity compared with the plasmid containing the complete promoter sequence. However, deletion of sequences from −571 bp and beyond completely abrogated luciferase activity compared with cells transfected with unmutagenized promoter sequences. Furthermore, the luciferase activities of these constructs was comparable with the activity observed in unstimulated cells and in cells transfected with the control plasmid (pGL3B) (Fig. 5). Similar results were obtained for both THP-1 (Fig. 5, middle panel) and THP-1/CD14 (Fig. 5, right panel) cells. The results shown are a mean of four experiments performed with each of the THP-1 and THP-1/CD14 cells transfected with the hIL-10 promoter constructs containing 5′-deletions. These results suggest that DNA sequences located between −571 and −652 bp relative to the +1 transcription site are necessary for hIL-10 transcription in THP-1 and THP-1/CD14 cells following LPS stimulation.

Sp1 Binding Site in the hIL-10 Promoter Is Sufficient to Induce IL-10 Production—A computer-aided analysis of the hIL-10 promoter sequence between −652 and −571 bp revealed the existence of consensus sequences for four transcription factors, including Sp1 (5′-ccccg-3′ at −636 to −631 bp), PEA1 (5′-aggaag-3′ at −622 to −617 bp), YY1 (5′-aatagggaa-3′ at −600 to −592), and ERNA-2-like factor (5′-ctgggatac-3′ at −585 to −575) (Fig. 3). This suggests that any one or more of the above mentioned transcription factors may be involved in regulating transcription of the hIL-10 gene. To investigate the role of the Sp1 transcription factor in hIL-10 gene transcription, we used PCR to introduce site-directed mutations in the Sp1 consensus sequence by substituting cytosine with guanine at position −631 bp and cytosine with adenine at position −636 bp (Fig. 6A). The fragment containing the Sp1 mutant sequence was cloned into pGL3B. To understand the role of ERNA-2-like transcription factor, we amplified another fragment spanning a distance from −589 to +120 bp and cloned it into pGL3B. This fragment was devoid of Sp1, PEA1, and YY1 transcription factor-binding sites. THP-1 and THP-1/CD14 cells transfected with a plasmid containing an ERNA-2-like transcription factor sequence did not show any increase in luciferase activity (Fig.

with LPS induced IL-10 expression as determined by enzyme-linked immunosorbent assay (Fig. 2A) and reverse transcriptase-based PCR analysis (Fig. 2B, C). Maximal levels of IL-10 mRNA were detected within 4 h after stimulation of THP-1 (Fig. 2B) and THP-1/CD14 (Fig. 2C) cells. Production of IL-10 protein was elevated in THP-1/CD14 cells compared with THP-1 cells, an observation that correlated with CD14 surface expression. Production of IL-10 was dependent on the concentration of LPS used for cell stimulation, and 1 μg of LPS produced maximal levels of IL-10 (data not shown).

Determination of DNA Sequences in the IL-10 Promoter Region Required for IL-10 Transcription—Inducible genes, including cytokine genes, contain DNA sequences within their promoter region that are responsible for regulating transcription. The human IL-10 promoter was recently cloned and characterized (41). To understand the regulation of IL-10 gene transcription in LPS-stimulated THP-1 cells, we used PCR to clone the hIL-10 promoter fragment encompassing nucleotide residues from −890 to +120 bp relative to the +1 transcription site (Fig. 3). The amplified promoter fragment was subcloned into the XhoI polylinker site of the luciferase reporter plasmid, pGL3B. THP-1/CD14 cells were transiently transfected with the amplified product and the location of consensus sequences for various transcription factors identified within the hIL-10 promoter (Fig. 3) are depicted in Fig. 5 (left panel). Examination of the DNA sequences within the hIL-10 promoter region containing various deletions revealed that deletion of sequences from −890 to −652 bp had no effect on luciferase activity compared with the plasmid containing the complete promoter sequence. However, deletion of sequences from −571 bp and beyond completely abrogated luciferase activity compared with cells transfected with unmutagenized promoter sequences. Furthermore, the luciferase activities of these constructs was comparable with the activity observed in unstimulated cells and in cells transfected with the control plasmid (pGL3B) (Fig. 5). Similar results were obtained for both THP-1 (Fig. 5, middle panel) and THP-1/CD14 (Fig. 5, right panel) cells. The results shown are a mean of four experiments performed with each of the THP-1 and THP-1/CD14 cells transfected with the hIL-10 promoter constructs containing 5′-deletions. These results suggest that DNA sequences located between −571 and −652 bp relative to the +1 transcription site are necessary for hIL-10 transcription in THP-1 and THP-1/CD14 cells following LPS stimulation.

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Figure 3. Nucleotide sequence of the 5′-flanking promoter region of hIL-10 gene (GenBank™ accession number X78437). The translation start codon (ATG) is italicized. The putative cis-regulatory elements are marked with solid lines below/above according to their orientation, respectively.
stimulated cells or cells transfected with the control plasmid (Fig. 9). Treatment of the same cells with p38 inhibitor SB202190 completely abrogated the luciferase activity (Fig. 9), suggesting the involvement of p38 in the regulation of Sp1 activity. As observed above, deletion of sequences between −571 and +120 bp did not show any increase in luciferase activity that remained comparable with the activity observed in unstimulated or in cells transfected with the control plasmid (pGL3B). Similar results were obtained with THP-1 cells transfected with the above mentioned plasmids containing hIL-10 promoter sequences with 5′-end deletions and cultured in the presence of p38 inhibitor (Fig. 9).

To confirm that p38 MAP kinase activates Sp1 transcription factor, THP-1 and THP-1/CD14 cells were transfected with a plasmid containing a wild type or mutant Sp1 sequence and cultured in the presence or absence of SB202190. Treatment with SB202190 completely abrogated the luciferase activity observed in LPS-stimulated cells transfected with the plasmid containing the wild type Sp1 sequence (Fig. 10). Similarly, treatment of LPS-stimulated cells transfected with plasmids containing either a mutated Sp1 sequence or an EBNA-2-like transcription factor sequence with p38 inhibitor SB202190 did not result in any change in luciferase activity (Fig. 10). The results shown are a mean of four experiments performed with both THP-1 (data not shown) and THP-1/CD14 cells (Fig. 10).

**Sp1 Binding to the IL-10 Promoter in LPS-stimulated Cells**

Regulation of hIL-10 Promoter by Sp1 and p38 MAP Kinase—To further understand which signaling events downstream of MAP kinases may be involved in IL-10 transcription, we investigated the activation of p38 MAP kinase substrates. The above results suggest that the p38 MAP kinase and the Sp1 play a role in the regulation of hIL-10 transcription. Therefore, we investigated whether LPS stimulation of THP-1 cells induced the binding of Sp1 to the Sp1 binding site in the hIL-10 promoter. Cells were stimulated with LPS over a period of time ranging from 0 to 240 min, and the nuclear extracts were analyzed in a gel shift assay for binding to Sp1 oligonucleotide probes. The results revealed that the maximum binding of Sp1 to the Sp1 oligonucleotide sequence of the hIL-10 promoter occurred 30–45 min following stimulation of THP-1/CD14 cells with LPS (Fig. 11A). We observed three distinct Sp1 DNA-protein complex bands, namely A, B, and C, in a gel shift assay that were completely blocked by competition with cold Sp1 oligonucleotides, indicating their specificities. It should be pointed out that bands A and B were always induced by LPS. However, induction of band C was not observed at all time points. The possible reasons for inconsistent induction of band C are not known. Similar results were obtained with LPS-stimulated THP-1 cells (data not shown). To determine whether p38 MAP kinase delivers a signal via the activation of Sp1 transcription factor, we investigated whether SB202190, an inhibitor of p38 MAP kinase, inhibits binding of Sp1 to the Sp1-binding site of the IL-10 promoter. Incubation of THP-1/CD14 cells with SB202190 for 2 h prior to stimulation with LPS resulted in the inhibition of Sp1 binding to the oligonucleotide containing the Sp1 sequence (Fig. 11B). As above, p38 inhibitors significantly reduced Sp1 binding to its oligonucleotides in bands A and B. In contrast, PD98059 did not affect Sp1 binding in LPS-stimulated cells (data not shown). These results suggest that p38 MAP kinase may promote IL-10 expression by activating Sp1.

**DISCUSSION**

Bacterial endotoxin (LPS) is responsible for many of the cellular responses to Gram-negative bacterial infections (43). These responses may be induced after the association of LPS with the LPS-binding plasma protein and the binding of this complex with the CD14 receptor expressed on cells of monocytic lineage (44). LPS stimulates a variety of cytokines including proinflammatory (IL-1, IL-6, TNF-α, etc.) and anti-inflammatory cytokines (e.g., IL-10) (7, 31, 33, 43). It is believed that LPS may stimulate the expression of proinflammatory cytokines through a common signaling pathway during inflammation. There is reasonably good evidence that production of TNF-α, IL-1β, and IL-6 can be regulated by the transcription factor NF-κB in various cell types (33–37). However, little is known concerning the regulation of hIL-10. The lack of xB binding sites in the hIL-10 promoter makes it unlikely that the NF-κB is involved in IL-10 regulation (41). In this study, we investigated the regulation of the hIL-10 promoter in a human mononuclear cell line, THP-1, following activation with LPS. Extensive deletion analysis of hIL-10 promoter sequences revealed that an element encompassing the Sp1 transcription factor-binding site is essential for IL-10 transcription. This was confirmed by transfecting THP-1 cells with a plasmid containing a mutated Sp1 site, which was unable to drive the expression of luciferase reporter. In addition, we analyzed the events upstream of Sp1 activation. Our results clearly demonstrate that p38 MAP kinase regulates the LPS-induced activa-
tion of Sp1, which in turn regulates the transcription of the hIL-10 gene. These data suggest that the molecular regulation of pro- and anti-inflammatory cytokine genes is differentially regulated through diverse transcription factors (7, 8, 33, 34).

To better understand the LPS-induced signaling pathway in the regulation of IL-10 synthesis, we employed two types of THP-1 cell lines that differ with respect to the level of CD14 receptor expression on their surfaces. CD14 was expressed on 5–15% of the THP-1 cells. To enhance LPS-mediated response in these cells, THP-1/CD14 cells were used; these cells constitutively expressed CD14. Hence, transfection of THP-1/CD14 cells with 5'-deletion mutants of hIL-10 promoter linked to the luciferase gene consistently revealed higher luciferase activity compared with THP-1 cells transfected with the same constructs. It should be pointed out that LPS stimulation of THP-1 cells induces cytokine production in a manner similar to that observed with normal human monocytes. However, THP-1 cells, unlike normal human monocytes, are free from negative feedback regulation mediated by endogenously produced IL-10 (data not shown).

Sp1 is a ubiquitous transcription factor that regulates the constitutive activity of many genes studied. Sp1 plays a vital role in the regulation of transcription from TATA-less promoters that commonly encode housekeeping genes (45). Sp1 activi-
ity and cellular content have been shown to be regulated during development (46, 47), cellular proliferation (48), apoptosis (49), and other cellular processes (50, 51). Sp1 has been shown to be involved in mediating responses to various stimuli including induction of the TGF-β receptor gene (51, 52), epidermal growth factor-mediated expression of the gastrin gene (53), and cAMP-dependent induction of the CYP11A gene (54).

How Sp1 mediates its responses is presently not fully understood. Sp1 is a well characterized protein composed of 778 amino acids. The amino-terminal portion of the molecule contains two glutamine-rich domains, each of which is associated with serine/threonine-rich regions (55). These domains are involved in transcriptional activation. The C-terminal region of the molecule contains the zinc finger DNA-recognition domain (55). Most of the gene regulation mediated by Sp1 requires either post-translational modifications of Sp1, such as phosphorylation and glycosylation (46, 56, 57) or alterations in the abundance of Sp1 protein (51). In addition, there are several coactivators of Sp1 such as CRSP, Rb, and hTAFII 130, which allow Sp1 to stimulate transcription very effectively (45, 58).

Translational modification of any of these coactivators may

![Image](image_url)

**FIG. 7.** LPS stimulation induces p38 and p42/44 MAP kinase activity in THP-1/CD14 cells. To determine the effects of the p38 MAP kinase-specific inhibitor (SB202190) and the p42/44 MAP kinase-specific inhibitor (PD98059) on LPS-induced activation of p38 or p42/44 MAP kinase, respectively, cells were treated with SB202190 or PD98059 for 2 h. Cells were then stimulated with LPS (1 μg/ml) for 20 min, followed by centrifugation and lysis of cell pellets. Proteins from the cell lysates were immunoprecipitated with anti-p38 (A) and anti-p42/44 (B) rabbit polyclonal antibodies (Santa Cruz Biotechnology). The immune complexes were subjected to SDS-polyacrylamide gel electrophoresis followed by transfer of proteins onto the membranes. The membranes were blotted with anti-phosphotyrosine antibodies (apy). To control for protein loading, the membranes were stripped and reprobed with the same antibody used for immunoprecipitation. Ab, a control immunoprecipitation performed with antibody and Sepharose beads in the absence of lysate. The experiment shown is representative of three experiments.

![Image](image_url)

**FIG. 8.** LPS-stimulated IL-10 production is selectively inhibited by inhibitors of p38 MAP kinases. To determine the effects of the p38 MAP kinase-specific inhibitors, SB203980 and SB202190, and the p42/44 MAP kinase-specific inhibitor PD98050 on LPS-induced IL-10 production, cells were treated with inhibitors for 2 h prior to stimulation with LPS (1 μg/ml). The supernatants were harvested after 48 h and analyzed by enzyme-linked immunosorbent assay for IL-10 production. The experiment shown is representative of three experiments.

![Image](image_url)

**FIG. 9.** Effect of the p38 inhibitor SB202190 on LPS-induced hIL-10 promoter activation in THP-1/CD14 cells. Cells (1.5 × 10^6) were transiently cotransfected with 10 μg of either hIL-10 wild type promoter or its deletion constructs and with 5 μg of β-galactosidase control vector. The transfected cells were pretreated with 15 μM SB202190 for 2 h followed by treatment with 1 μg/ml of LPS for 24 h. Unstimulated, LPS-stimulated, and LPS + SB202190-treated cells were harvested, and their lysates were assessed for luciferase and β-galactosidase activities. The results shown are means ± S.D. of three experiments performed in triplicate and normalized by β-galactosidase activity.
modulate the ability of Sp1 to regulate transcription. Molecular mechanisms by which Sp1 regulates IL-10 transcription remain to be investigated.

It was surprising to find that only one transcription factor (Sp1) seems to play a prominent role in IL-10 regulation. This is in contrast to most of the cellular genes, and especially cytokine genes, that are regulated by multiple transcription factors. In view of established models of multiple transcription factor involvement, it seems unlikely that other transcription factors are not involved in the regulation of the hIL-10 gene. Our studies do not rule out the involvement of other transcription factors that may cooperate with Sp1 in hIL-10 transcription. It is likely that the transient transfection assay used in the current study may not reveal the involvement of other transcription factors. These factors may remain masked in our experimental system of transient transfection, a system that is known to generate a high plasmid copy number or the accumulation of aberrant chromatin structure in these cells (59). To circumvent this possibility, a stable transfection approach may be required. It is also possible that other transcription factors interacting with the hIL-10 promoter region beyond the −890 bp region participate in regulating the transcription of the hIL-10 gene. Further studies are required to address this possibility. It is also likely that other transcription factors may be involved in IL-10 gene regulation in different IL-10-producing cell types and in response to distinct stimuli.

We have also investigated the upstream signaling events that lead to Sp1 activation. We primarily investigated the role of MAP kinases in this process and in IL-10 production via stimulation of CD14 receptors in THP-1 cells. LPS has been shown to activate p38, p42/44 ERK, and JNK MAP kinases (24). These three types of MAP kinases can be activated individually or simultaneously, thereby suggesting their independent signaling roles (24). The data presented in this study show the selective involvement of p38 in IL-10 production in THP-1 cells. Expression of the luciferase gene linked to the hIL-10 promoter region was mediated via p38 MAP kinase activation. A specific inhibitor of the p42/44 ERK MAP kinases did not affect luciferase activity (data not shown). In addition, we also demonstrated that LPS induces the activation of Sp1 transcription factor in a time-dependent manner. Maximum activity of Sp1 was observed at 30–60 min poststimulation with LPS. Similarly, p38 inhibitors significantly reduced Sp1 binding to its oligonucleotides. Furthermore, using Sp1 mutants of the hIL-10 promoter linked to the luciferase reporter gene, we demonstrate for the first time that p38 MAP kinase plays a direct role in Sp1 activation and in inducing Sp1 binding to the IL-10 promoter.

The LPS-induced signaling pathway leading to the activation of the p38 MAP kinase in monocytes/macrophages has been investigated. LPS signaling through CD14 has been shown to involve Toll-like receptors (TLR), specifically TLR-4, which associates with CD14 (60–62). LPS interaction with CD14 promotes dimerization of the TLR and subsequent recruitment of MyD88, a myeloid differentiation marker that functions as an adaptor molecule (62, 63). MyD88 associates via its c-terminal toll homology domain with TLR and via its N-terminal death domain with a serine-threonine protein kinase, IL-1R associated kinase (63). Upon interaction with MyD88, IL-1R-associated kinase is autophosphorylated and binds to TRAF-6 (TNF-receptor associated factor-6). TRAF-6 subsequently activates TAK-1 and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase, which activates MEK, JNK, and p38 MAP kinase (64–65). The molecular mechanism by which p38 MAP kinase activates Sp1 to induce IL-10 gene transcription remains to be investigated. Nonetheless, the p38 MAP kinase has been implicated in the activation of Sp1 in IL-1β-induced vascular endothelial cell growth factor gene expression (66) and in hyperosmotic stress-regulated cellular utilization of the serum- and glucocorticoid-inducible protein kinase (Sgk) (67).

Activation of the p38 MAP kinase has been shown to play a critical role in the regulation of several cellular and cytokine genes in T cells and monocytes following their stimulation with various ligands. For example, T cell activation with specific antigen or staphylococcal antigens has been shown to induce p38 activation, resulting in TNF-α production (25). It has also been demonstrated that p38 MAP kinase regulates IL-1 (25), IL-6 (68), TNF-α (25), IL-10 (31), and prostaglandin H synthase-2 (69) production in human monocytes through the activation of the CD14 receptor. Functional roles for p38 have also been described. The p38 MAP kinase is constitutively active in mouse thymocytes, suggesting a role in T cell survival (70, 71). Antigen receptor or Fas-mediated apoptosis of T and B cells is accompanied by p38 activation (72, 73).

In summary, our results clearly show for the first time the involvement of the Sp1 transcription factor, and its activation via p38 MAP kinase, in the regulation of hIL-10 gene transcription in human monocytic cell lines. While this work was in progress, Brightbill et al. (74) demonstrated the involvement of a nonconsensus Sp1-like sequence in mIL-10 expression using RAW264.7, a murine macrophage cell line. Although the hIL-10 gene bears >80% nucleotide sequence homology and >73% amino acid sequence homology with mIL-10, the mIL-10 and hIL-10 genes and their promoters are distinct (1–3). In contrast to mIL-10, hIL-10 cDNA clones contain the insertion of Alu repetitive sequence elements in the 3′ untranslated region (2). Furthermore, the Sp1 consensus sequence is not present in the mIL-10 promoter (74). In contrast to our findings,
LPs stimulation of murine monocyctic cells did not result in Sp1 activation (74), indicating perhaps differential regulation of IL-10 synthesis in murine and human monocyctic cells. Taken together, our results point to the key role of Sp1 and its activation via p38 MAP kinase in the regulation of IL-10 transcription. These studies may provide a basis for the identification of molecular players in IL-10 regulation that may help in designing targeted drug therapy for inflammatory diseases.

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**FIG. 11.** LPS stimulation activates Sp1 transcription factor activity in a time-dependent manner (A), and Sp1 activity is inhibited by inhibitors of p38 MAP kinase (B). A, THP-1 macrophages were treated with LPS (1 μg/ml) for various times ranging from 15 min to 4 h followed by centrifugation and collection of cell pellets. B, to determine the effects of the p38 MAP kinase-specific inhibitor, SB202190, on LPS-induced Sp1 activity, cells were treated with SB202190 for 2 h prior to stimulation with LPS (1 μg/ml). To perform the gel shift assay, nuclear extracts were harvested from the cell pellets obtained at each time point. Nuclear extracts containing 5 μg of proteins were incubated for 1 h with 32P-labeled oligonucleotides corresponding to the consensus sequence for Sp1. To determine the specificity of Sp1 transcription factor binding, the nuclear extracts were incubated with either unlabeled oligonucleotides, corresponding to the consensus sequence for Sp1, or with the control base pair-matched irrelevant oligonucleotide. The complexes were subjected to electrophoresis followed by autoradiography. Three distinct Sp1 DNA-protein complex bands, namely A, B, and C, were completely blocked by competition with cold Sp1 oligonucleotides, indicating their specificities.
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