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Regulation of B7.1 Costimulatory Molecule Is Mediated by the IFN Regulatory Factor-7 through the Activation of JNK in Lipopolysaccharide-Stimulated Human Monocytic Cells

Wilfred Lim,† Katrina Gee,† Sasmita Mishra,‡ and Ashok Kumar2*†‡

The engagement of CD28 or CTLA-4 with B7.1 provides the essential second costimulatory signal that regulates the development of immune responses, including T cell activation, differentiation, and induction of peripheral tolerance. The signaling molecules and the transcription factors involved in B7.1 regulation are poorly understood. In this study we investigated the role of MAPKs in the regulation of LPS-induced B7.1 expression in human monocytes and the promonocytic THP-1 cells. Our results show that LPS-induced B7.1 expression in monocytic cells did not involve the activation of either p38 or ERKs. Using the JNK-specific inhibitor SP600125, small interfering RNAs specific for JNK1 and JNK2, and agents such as dexamethasone that inhibit JNK activation, we determined that LPS-induced B7.1 expression was regulated by JNK MAPK in both monocytes and THP-1 cells. In addition, we identified a distinct B7.1-responsive element corresponding to the IFN regulatory factor-7 (IRF-7) binding site in the B7.1 promoter responsible for the regulation of LPS-induced B7.1 transcription. Furthermore, SP600125 and dexamethasone inhibited LPS-induced IRF-7 activity. Taken together, these results suggest that LPS-induced B7.1 transcription in human monocytic cells may be regulated by JNK-mediated activation of the IRF-7 transcription factor. The Journal of Immunology, 2005, 175: 5690–5700.

The family of B7 costimulatory molecules consists of at least two members, B7.1 (CD80) and B7.2 (CD86) (1, 13). B7.1 and B7.2 have also been shown to have opposing functions through CD28 and CTLA-4 on the immunosuppressive activities (9, 10, 19–21), perhaps due to their distinct expression kinetics and binding affinities to their receptors. In addition to CD28, both B7.1 and B7.2 can bind to CTLA-4 (CD152). Recent evidence suggests that B7.1 is a more potent ligand for CTLA-4 based on its higher affinity and avidity. B7.1, in contrast to B7.2, binds 2–3 times more strongly to both CD28 and CTLA-4, with faster binding kinetics and slower dissociation constants (22–24). Interaction of B7.1 or B7.2 with CTLA-4 results in an inhibitory signal, in contrast to the positive signal generated after its interaction with CD28 (25). B7.1 and B7.2 have also been shown to have opposing functions through CD28 and CTLA-4 on the immunosuppressive activity of regulatory T cells (26) and exhibit distinct immunological effects in a number of autoimmune diseases, such as experimental autoimmune encephalomyelitis and the NOD model of diabetes (9, 10, 27).

In light of the influence of B7 expression on immune functions, alterations in B7.1 expression may have profound effects on immune responsiveness and disease progression. Therefore, understanding the mechanisms and characterization of signal transduction events regulating B7.1 expression may facilitate the design of strategies for treatment of autoimmune diseases and cancer. Very little is known about the signal transduction pathways involved in B7.1 regulation (28, 29). In one study a cell type-specific, 183-bp enhancer element 3 kb upstream of the transcription start site of the human B7.1 gene was found to be responsive to stimulation with two distinct stimuli, LPS and dibutyryl cAMP, both known to regulate B7.1 expression (28). Site-directed mutagenesis of this region revealed the presence of an NF-kB consensus sequence that was consequently implicated in B7.1 transcription. Interestingly,
the promoter region immediately upstream of the transcription start site was found to be inactive unless linked with its native enhancer element (28). Subsequently, Fong et al. (29) identified a positive B7 regulatory element (B7-RE), located at –60 to –47 bp upstream of the transcription start site that regulated B7.1 transcription. However, the identity of the transcription factor binding to the B7.1 RE was not established.

In this study we have investigated the regulation of B7.1 expression in primary human monocytes and the promonocytic THP-1 cells in response to stimulation with LPS, a bacterial cell wall component and an important contributor to sepsis. We investigated the role of MAPKs because they play a key role in cellular responses such as proliferation, differentiation, and apoptosis (30). The three main families of MAPKs are the ERKs, the JNKs, and the p38 MAPKs (30). LPS is known to activate all three types of MAPKs (30, 33). LPS-derived from Salmonella, p38 MAPKs (35). LPS is known to activate all three types of MAPKs (30, 34), was also purchased. All other chemicals were purchased from Sigma-Aldrich.

Materials and Methods

Cell line, cell culture, and reagents

THP-1, a promonocytic cell line derived from a human acute monocytic leukemia patient, was obtained from the ATCC (American Type Culture Collection). THP-1, stably transfected with a plasmid containing CD14 cDNA sequences (THP-1/CD14) were provided by Dr. R. Ulevitch (The Scripps Research Institute, La Jolla, CA) (32). Cells were cultured in IMDM (Sigma-Aldrich) supplemented with 10% FBS (Invitrogen Life Technologies), 100 U/ml penicillin, 100 μg/ml gentamicin, 10 mM HEPES, and 2 mM glutamine. PD98059, an inhibitor of MAP/ERK kinase-1 kinase that selectively blocks the activity of ERK MAPK (30, 33), was purchased from Calbiochem. Pyridinyl imidazole, SB202190, a potent inhibitor of p38 MAPK without any effect on ERK or JNK MAPK activity (30, 34), was also purchased. All other chemicals used for electrophoresis and immunoblot analysis were obtained from Sigma-Aldrich.

Table I. Primer sequences used to amplify the B7.1 promoter fragments from genomic DNA

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Region Amplified (bp)</th>
<th>Product Length (bp)</th>
</tr>
</thead>
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<td>Sense primers</td>
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<tr>
<td>B7-1 (+587)</td>
<td>5′-GGGTACCGAATCTTAAATCTTCAGAGACCA-3′</td>
<td>587/+57</td>
<td>644</td>
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<tr>
<td>B7-1 (+403)</td>
<td>5′-GGGTACCGAATCTTAAATCTTCAGAGACCA-3′</td>
<td>−403/+57</td>
<td>460</td>
</tr>
<tr>
<td>B7-1 (+225)</td>
<td>5′-GGGTACCGAATCTTAAATCTTCAGAGACCA-3′</td>
<td>−225/+57</td>
<td>282</td>
</tr>
<tr>
<td>B7-1 (+84)</td>
<td>5′-GGGTACCGAATCTTAAATCTTCAGAGACCA-3′</td>
<td>−84/+57</td>
<td>141</td>
</tr>
<tr>
<td>B7-1 (+84)Mutant1</td>
<td>5′-GGGTACCGAATCTTAAATCTTCAGAGACCA-3′</td>
<td>−84/+57</td>
<td>141</td>
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<tr>
<td>B7-1 (+84)Mutant2</td>
<td>5′-GGGTACCGAATCTTAAATCTTCAGAGACCA-3′</td>
<td>−84/+57</td>
<td>141</td>
</tr>
<tr>
<td>B7-1 (+72)</td>
<td>5′-GGGTACCGAATCTTAAATCTTCAGAGACCA-3′</td>
<td>−72/+57</td>
<td>129</td>
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<td>B7-1 (+28)</td>
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<td>−28/+57</td>
<td>85</td>
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<td>Antisense primer</td>
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<tr>
<td>B7-1 (+57)</td>
<td>5′-CGCATGTCCTTAATCTTCAAGACGACAT-3′</td>
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<td></td>
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</tbody>
</table>

Isolation of monocytes from PBMCs

Blood was obtained for isolation of PBMCs from healthy volunteers after approval of the protocol by the ethics review committee of Children’s Hospital of Eastern Ontario. PBMCs were isolated by density gradient centrifugation over Ficoll-Hypaque (Pharmacia Biotech) as previously described (36). The cell layer consisting mainly of mononuclear cells was collected and washed three times in PBS. Briefly, purified nonactivated monocytes were isolated by negative selection after depletion of T and B cells using magnetic polystyrene Dynabeads coated with anti-CD2 (T cells) and anti-CD19 (B cells) Abs (Dynal Biotech) as previously described (36). CD2+CD19+ cells were separated magnetically from CD2+CD19− cells. CD2+CD19− cells were incubated at 37°C for 2 h, after which nonadherent cells were removed. The adherent cells thus obtained contained <1% T and B cells, as determined by flow cytometry.

Cell stimulation

To determine the effect of MAPK inhibitors on B7.1 expression, monocytes (0.5 × 10⁶ cells/ml) and THP-1/CD14 cells (1 × 10⁶ cells/ml) were incubated with inhibitors specific for ERK, p38, or JNK MAPKs for 2 h, followed by stimulation with 1 μg/ml LPS in 24-well culture plates (Falcon; BD Biosciences) for 0–15 min for Western blot analysis and for 24 h for analysis of B7.1 expression by flow cytometry.

Flow cytometry

B7.1 expression was measured by flow cytometry as described previously (15, 37). Briefly, cells were double stained with 3 μl of FITC-labeled anti-CD14 mAb, and 3 μl of PE-conjugated anti-B7.1 mAb (BD Pharmingen). Anti-CD19 and isotype-matched control Abs, IgG1(κ) (BD Biosciences), were also included. Analysis of B7.1 expression was performed on CD14+ monocytes. Data were acquired on a FACSScan flow cytometer (BD Biosciences) and were analyzed using the WinMDI version 2.8 software package (J. Trotter, The Scripps Institute, San Diego, CA). The validity of comparisons in the expression levels of CD14 and B7.1 between different samples was ensured through the use of Calibrite beads (BD Biosciences).

Western blot analysis

Phosphorylation of p38, p42/44 ERK, and JNK MAPKs was determined by Western blot analysis using phospho-MAPK-specific Abs according to the standard procedures, as previously described (32, 36). Briefly, total cell proteins obtained after lysis of cell pellets recovered from LPS-stimulated cells were subjected to 10% SDS-PAGE. The proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad). The membranes were probed with rabbit anti-phospho-p38 (Cell Signaling Technology), mouse anti-phospho-p42/44 (Santa Cruz Biotechnologies), or rabbit anti-phospho-JNK Abs (Cell Signaling Technology), followed by HRP-conjugated goat anti-rabbit or goat anti-mouse polyclonal Abs (Bio-Rad). The membranes were stripped of the primary Abs and reprobed with rabbit polyclonal Abs specific for the unphosphorylated forms of p38, p42, or JNK MAPKs (Santa Cruz Biotechnologies). All immunoblots were visualized by ECL (Amersham Biosciences).

RNA isolation and semiquantitative RT-PCR analysis for B7.1

Total RNA was extracted using a monophasic solution containing guani
dine thiocyanate and phenol (Tri-Reagent solution; Molecular Research
cystic cells were stimulated with LPS (1 ng/ml) for 24 h, followed by analysis of B7.1 expression by flow cytometry. The experiment shown is representative of three experiments.

FIGURE 1. LPS up-regulates B7.1 expression in monocytes. A, Purified human monocytes (0.5 × 10^6/ml) were stimulated with LPS (1 μg/ml) for 24 h, followed by analysis of B7.1 expression by flow cytometry. B, Monocytic cells were stimulated with LPS (1 μg/ml) for 0–12 h. B7.1 expression was determined by semiquantitative RT-PCR analysis using B7.1 antisense and /H11032-actin as a standard control. The experiment shown is representative of three experiments.

Center) as described previously (32). Total RNA (1 μg) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (PerkinElmer). Equal aliquots (5 μl) of cDNA were subsequently amplified for B7.1 and β-actin. The oligonucleotide primer sequences used for B7.1 and /H9252-actin were as follows: B7.1 sense, 5′-AGT ACA AGA GAC GAT GGA GGG-3′; and /H11032-actin antisense, 5′-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3′; /H11032-actin sense, 5′-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3′; and /H11032-actin antisense, 5′-GAC GAT GGA GGG-3′. The amplification conditions for B7.1 and /H11032-actin were as follows: denaturation at 94°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 1 min. After 30 cycles, the amplified B7.1 (605 bp) and β-actin (661 bp) fragments were resolved by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining.

Transfection of cells with JNK1 and JNK2 stealth small interfering RNA (siRNA)

THP-1/CD14 cells were resuspended at a concentration of 1 × 10^5 cells/well in a 96-well plate (Falcon; BD Biosciences) in a total volume of 100 μl. The cells were transfected with 50 pmol of stealth RNA (Invitrogen Life Technologies) specific for either JNK1 or JNK2 using FuGene (Roche) according to the manufacturer’s directions. Briefly, FuGene and the stealth siRNA were incubated in serum-free IMDM for 15 min at room temperature and then were added dropwise to the cell culture to a final volume of 200 μl. The cells were cultured for 24 h, followed by stimulation with LPS (100 ng/ml) for 8 h. Cells were then harvested and analyzed for B7.1 expression by flow cytometry. The transfection efficiency was determined to be 15% through the use of BLOCK-IT fluorescent oligo that was included in the stealth siRNA assay kit.

EMSAs

EMSAs were performed according to the standard technique and as described previously (32). Briefly, cells (10^6) harvested in Tris-EDTA-saline buffer (pH 7.8) were lysed for 10 min at 4°C with buffer A (10 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT, and 0.5 mM phenylmethylsulfonylfluoride (pH 7.9) containing 0.1% Nonidet P-40). The lysates were centrifuged at 20,000 × g for 10 min at 4°C. The pellet containing the nuclei was resuspended in buffer B (20 mM HEPES, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, and 25% glycerol) at 4°C for 15 min, and both buffers A and B contained DTT and phenylmethylsulfonylfluoride. Buffer B also contained sperridine (0.5 mM), spermine (0.15 mM), and 5 μl/ml of each of the proteolytic inhibitors aprotinin, leupeptin, and pepstatin. Nuclear extracts (5 μg) were mixed with 32P-labeled B7.1 oligonucleotide probes for 20 min at room temperature. The oligonucleotide sequences corresponding to the B7.1 (~90–67) binding sites in the B7.1 promoter were as follows: 5′-(TAC AAA AGC TTT TCG TTC CTG)-3′ and 3′-(ATG TTT TTC CCC TTC ATC TTC TTC-5′). Positive regulatory domain I (PRD-I) oligonucleotides containing IRF-7 consensus sequences were as follows: 5′-(TAC AAA AGC TAG AAG AAG ACG)-3′ and 3′-(ATG TTC ATC TTT AC TTT TC-5′). To illustrate specificity of NF binding to labeled probes, parallel

FIGURE 2. LPS-induced B7.1 expression in human monocytes is not regulated by p38 or ERK MAPks. A, Purified monocytes (0.5 × 10^6/ml) were treated with either SB202190 or PD98059 at concentrations ranging from 0 to 50 μM for 2 h before LPS (1 μg/ml) stimulation for 10 min. Total proteins (50 μg) were analyzed by Western blot analysis using either anti-phospho-p38 (pp38) or anti-phospho-p42/44 (pp42/44) Abs. To control for equal protein loading, the membranes were stripped and reprobed with either anti-p38 or anti-p42/44 Abs, respectively. B, Monocytes were treated with either SB202190 or PD98059 at concentrations ranging from 0 to 50 μM for 2 h before stimulation with LPS (1 μg/ml) for 24 h, followed by analysis of B7.1 expression by flow cytometry. The experiment shown is representative of three experiments.
EMSA reactions were incubated with a 100- to 400-fold excess of cold unla-
beled oligonucleotides and nonspecific NF-
B probes (Promega). The bound
and unbound32P-labeled probes were resolved by nondenaturing 5% PAGE.
The gel was dried and exposed to x-ray film (Eastman Kodak).

Construction of luciferase reporter gene vectors

Luciferase reporter gene vectors containing the human B7.1 promoter frag-
ment (600 to 50; GenBank accession no. U33208) were constructed as
described previously (32). A series of B7.1 promoter fragments was am-
plified from genomic DNA by PCR using primers containing
Kpn1 r NheI and
BglII restriction sites. The sequences of primers used for amplification
are listed in Table I. The amplification consisted of 30 cycles of denatur-
ation at 95°C for 1 min, annealing at 62°C for 1 min, and final extension
at 72°C for 1 min. To introduce mutations in the B7.1 promoter, site-
directed mutagenesis was performed by PCR using mutagenic primers. The
amplified products were inserted into the pCRII TOPO vector using the
TOPO TA cloning kit (Invitrogen Life Technologies). The sequences were
confirmed, and the correct insertions were subcloned into the
Kpn1 r NheI and
BglII polylinker sites of the luciferase reporter plasmid, pGL3E (Pro-
mega). DNA sequencing was performed by the Biotechnology Research
Institute, University of Ottawa.

Transient DNA transfection and measurement of luciferase
activity

For transient transfections, plasmids were isolated and purified using the Qia-
gen Endofree Plasmid Maxi Kit. Cells were transiently transected using
DEAE-dextran. Cells (5 × 106/ml) were seeded into six-well plates at 37°C for
1 h. Five micrograms of reporter plasmid and 2.5 μg of pSV-β-galactosidase
control vector were mixed with 800 μg/ml DEAE-dextran in 1 ml of PBS
buffer and added to the cells. The cells were incubated at 37°C for 30 min,
washed twice with PBS, resuspended, and cultured for 24 h in complete
IMDM. After incubation, the transfected cells were stimulated with LPS (1
μg/ml) for another 24 h. Cells were harvested, and cell lysates were assayed
for firefly luciferase and β-galactosidase activities using luciferase and β-ga-
lactosidase assay kits, respectively (Promega), in a Bio Orbit 1250 lumi-
nometer (Fisher Scientific) as described previously (32). Luciferase activity
for each transfection was normalized against the β-galactosidase activity.

Statistical analysis

Means were compared by two-tailed Student’s t test. The results are ex-
pressed as the mean ± SD.

Results

LPS-induced B7.1 expression involves activation of JNK MAPks
in human monocytes and THP-1/CD14 cells

We confirmed our previous observations (15) that LPS induces
B7.1 expression on primary monocytes as determined by flow cy-
motometry and RT-PCR analysis (Fig. 1). To understand the signaling
pathway involved in B7.1 regulation, we first investigated the roles of p38 and ERK MAPKs in LPS-stimulated monocytes. Monocytes isolated from healthy individuals were stimulated with LPS for 10 min and subjected to Western immunoblotting for p38 and ERKs activation using anti-phospho-p38 and anti-phospho-p42/44 ERK-specific Abs, respectively. The same blots were stripped and reprobed with anti-JNK Abs, B and C, THP-1/CD14 cells (1 × 10^6/ml) were treated with either SP600125 or DXM for 2 h before stimulation with LPS (1 μg/ml) for 4 h, followed by semiquantitative RT-PCR analysis for B7.1 using β-actin as a standard control (B), or for 24 h, followed by analysis of B7.1 expression by flow cytometry (C). The experiment shown is representative of three experiments.

FIGURE 5. LPS-induced B7.1 expression in THP-1/CD14 cells is regulated by JNK MAPKs. A, THP-1/CD14 cells (1 × 10^6/ml) were treated with various concentrations of SP600125 or DXM for 2 h before stimulation with LPS (1 μg/ml) for 15 min. Total proteins were analyzed for phosphorylation of JNK MAPK using anti-epoxy-JNK Ab. To control for protein loading, the membranes were stripped and reprobed with anti-JNK Abs. B and C, THP-1/CD14 cells (1 × 10^6/ml) were treated with either SP600125 or DXM for 2 h before stimulation with LPS (1 μg/ml) for 4 h, followed by semiquantitative RT-PCR analysis for B7.1 using β-actin as a standard control (B), or for 24 h, followed by analysis of B7.1 expression by flow cytometry (C). The experiment shown is representative of three experiments.

We next examined the role of JNK, the third major member of the MAPK family, using its specific inhibitor, SP600125 (35), and other agents, such as DXM and curcumin, that have been shown to block the activation of JNK MAPKs (38, 39). LPS induced the phosphorylation of JNK MAPKs, and its specific inhibitors, SP600125, DXM (Fig. 3A), and curcumin (data not shown), inhibited JNK phosphorylation in monocytes, respectively (Fig. 3A). To determine the roles of p38 and ERK MAPKs in LPS-induced B7.1 expression, we analyzed B7.1 expression in LPS-stimulated monocytes pretreated for 2 h with their specific inhibitors. B7.1 induction was not inhibited by either SB202190 or PD98059 at any concentration (Fig. 2B). Doses >50 μM for these inhibitors were not used, because these concentrations were cytotoxic, as determined by the propidium iodide assays (data not shown).

To confirm the involvement of JNK in the regulation of B7.1 expression, we used stealth siRNAs (Invitrogen Life Technologies) specific for JNK1 and JNK2 to knock down endogenous JNK expression. THP-1/CD14 cells were transfected with siRNA for JNK1, JNK2, or control siRNA for a period of 24 h, followed by LPS stimulation for 10 min. Transfection of cells with siJNK1 or siJNK2 RNAs significantly inhibited endogenous expression of JNK1, JNK2, respectively (Fig. 6A, upper panel). Because it is difficult to transfect a high proportion of THP-1 cells, and LPS, being a powerful mitogen, can induce B7.1 expression at very low concentrations (10 ng/ml), we stimulated cells transfected with various siRNA preparations with a lower LPS concentration (100 ng/ml) and for a short duration of 8 h, in contrast to 24 h used previously. Transfection of cells with siRNAs
specific for JNK1 and JNK2 significantly inhibited LPS-induced B7.1 expression compared with cells transfected with control siRNA (Fig. 6B).

Role of IRF-7 binding site within B7.1 promoter in LPS-induced B7.1 transcription

The human B7.1 promoter has been cloned and characterized (28, 29). To understand the regulation of B7.1 transcription, we amplified the B7.1 promoter fragment encompassing nucleotide residues from 5′ −587 to 3′ +57 bp relative to the transcription start site and subcloned into KpnI and BglII sites of the luciferase reporter plasmid, pGL3E. Cells were transiently transfected with the B7.1 promoter/luciferase reporter plasmid (pB7.1Pr-GL3E). Cells were stimulated with LPS after 24 h of transfection and cultured for 0−24 h, after which relative luciferase activity was assessed. The unstimulated cells exhibited basal luciferase activity that was 0−24 h, after which relative luciferase activity was assessed. The unstimulated cells exhibited basal luciferase activity that was 2- to 3-fold that of unstimulated cells and 10- to 12-fold of cells transfected with the pGL3E control vector. The increase in luciferase activity was detected by 3 h and peaked at 6 h after LPS stimulation (Fig. 7). The maximum increase in luciferase activity ranged from ~2- to 3-fold that of unstimulated cells and 10- to 12-fold that of cells transfected with the pGL3E control plasmid. The cells transfected with pGL3E alone did not show any increase in luciferase activity after LPS stimulation (data not shown). In subsequent experiments, we measured luciferase activity in cells stimulated with LPS for 6 h.

To identify the transcription factor binding sequences required for B7.1 transcription, we generated a series of B7.1 promoter fragments (from 5′ −587 to 3′ +57 bp) by successive deletions starting from the 5′ end. The exact size of the amplified product and the location of consensus sequences for different transcription factors identified within the B7.1 promoter are depicted in Fig. 8A. Transfection of cells with plasmids containing various deletions of the B7.1 promoter revealed that deletion of sequences from −587 to −84 bp did not affect luciferase activity after LPS stimulation compared with cells transfected with the entire promoter sequence. We consistently observed increased luciferase activity in cells transfected with the pB7.1Pr-GL3E promoter fragment containing −84 to +57 bp; however, this increase was not significant compared with cells transfected with the entire fragment. In contrast, deletion of sequences upstream of −72 bp significantly decreased luciferase activity (Fig. 8A), suggesting that DNA sequences located between −84 to −72 bp may be necessary for B7.1 gene transcription in LPS-stimulated THP-1/CD14 cells.

Using MatInspector Professional, a computer-aided analysis of the B7.1 promoter sequence between −84 to −72 bp revealed the existence of a sequence similar to the IRF-7 transcription factor binding site. To examine the role of IRF-7, we introduced mutations on the IRF-7 binding site by site-directed mutagenesis (Fig. 8A). The two amplified fragments containing IRF-7 mutations were cloned into pGL3E. Cells transfected with the mutant plasmids showed significant reduction in luciferase activity compared with cells transfected with the entire plasmid (Fig. 8B). These results suggest that the IRF-7 sequence between −84 to −72 bp of the B7.1 promoter may play a role in the regulation of LPS-induced B7.1 transcription in monocytic cells.

SP600125 down-regulates B7.1 expression by inhibiting IRF-7 activity

The results from the flow cytometry data suggest that LPS-induced B7.1 expression is regulated by the activation of JNK MAPKs (Figs. 3 and 5). To investigate whether LPS-induced B7.1 transcription is regulated by IRF-7 through JNK activation, we examined the effect of SP600125 on B7.1 promoter activity. THP-1/CD14 cells transiently transfected with pB7.1Pr-GL3E containing −84 to +57 bp sequences (pB7.1Pr−84 to +57/GL3E) were pretreated for 2 h with SP600125 or, as a control, with calphostin C, a protein kinase C inhibitor. As observed above, transfection of cells with pB7.1Pr−84 to +57/GL3E revealed an ~15-fold increase in luciferase activity in LPS-stimulated cells compared with cells transfected with the control plasmid (Fig. 9). Pretreatment of the same cells with SP600125 significantly decreased luciferase activity in a dose-dependent manner, whereas pretreatment with calphostin C did not have any effect (Fig. 9). As expected, transfection of cells with pB7.1Pr-GL3E containing sequences from −28 to +57 bp or pGL3E did not show any increase in luciferase activity.
To determine the role of JNK in the regulation of B7.1 transcription through the activation of IRF-7, we investigated whether LPS stimulation of THP-1/CD14 cells induced the binding of IRF-7 to its binding site present in the B7.1 promoter by the gel shift assay. Cells were stimulated with LPS over a period of time ranging from 0 to 5 h, and the cytoplasmic and nuclear extracts were analyzed for binding of IRF-7 transcription factors to the oligonucleotide probe corresponding to the IRF-7 binding site in the B7.1 promoter. In cytoplasmic extracts from unstimulated cells, constitutive binding of IRF-7 to the probe in the form of three bands was observed. However, this binding decreased significantly when cytoplasmic extracts obtained at different times after LPS stimulation were used. In contrast, significant binding of IRF-7 to the oligonucleotide probe using nuclear extracts occurred 2–4 h after LPS stimulation (Fig. 10A). To determine the specificity of these three bands, cold competition experiments were performed. The binding observed with the upper two bands was completely blocked by competition with their respective cold B7.1 (IRF-7) oligonucleotides. However, the intensity of the lower third band remained unchanged, indicating its nonspecific nature (Fig. 10B). Furthermore, cold nonspecific NF-κB oligonucleotides failed to compete for IRF-7 binding to its labeled oligonucleotide probe (Fig. 10B). To determine the specificity of IRF-7 binding, we used the PRD-I oligonucleotide probe as a cold competitor. The PRD-1 oligonucleotide sequence, present in the IFN-β promoter region, specifically binds to IRF-7, but not the IRF-3 transcription factors (41). DNA gel shift competition analysis using cold B7.1 (IRF-7) oligonucleotides competed for binding with the labeled PRD-1 probe (Fig. 10B), suggesting the specificity of the top IRF-7 bands.

To determine whether DXM and SP600125 inhibited binding of IRF-7 to its binding site in the B7.1 promoter, cells were treated with SP600125 or DXM for 2 h before LPS stimulation for 4 h, followed by analysis of IRF-7 binding to its corresponding oligonucleotide probe. The results show that both DXM and SP600125 inhibited the binding of IRF-7 to its probe in LPS-stimulated cells.

The lower nonspecific band was not inhibited by either SP-600125 or DXM (Fig. 10C). Taken together, the results suggest that B7.1 gene transcription may be regulated by a unique IRF-7 transcription factor(s) through JNK activation.

**Discussion**

LPS induces a cascade of signaling events after its association with the LPS-binding plasma protein and subsequent binding of the LPS/LPS-binding protein complex with the CD14/TLR4 complex expressed on cells of myelomonocytic lineage (42). The molecular mechanism, in particular, the signaling molecules and the transcription factors involved in the regulation of B7.1 expression, are poorly understood. In this study we have examined the roles of MAPKs and the transcription factors involved in LPS-induced expression of B7.1 in human monocytes and the promyelomonocytic THP-1 cells as a model system. Our results show that JNK MAPK plays a critical role in the regulation of LPS-induced B7.1 expression. In addition, we have identified for the first time a distinct B7.1-responsive element corresponding to the IRF-7 binding sequence located between −84 and −72 bp upstream of the transcription start site of the B7.1 gene. Furthermore, LPS-induced B7.1 expression in monocyctic cells may be regulated by the IRF-7 transcription factor through the activation of JNK MAPKs.

B7.1 expression can be induced by a number of stimuli, including IL-4, anti-BCR, and anti-CD40 Abs on B cells and stimulation of monocytic cells with LPS and IFN-γ (1, 15, 43). Because B7 plays a critical role in T cell activation and differentiation (5), immunoregulatory cytokines and mitogens, such as LPS, that enhance B7.1 expression provide a mechanism for the amplification of T cell activation and the development of cellular immune responses and autoimmune disorders. Therefore, understanding B7 regulation and characterizing the signal transduction events involved may lead to the development of strategies for the treatment of inflammation, autoimmune diseases, and cancer.

Very little is known regarding the signaling pathways and transcription factors involved in the regulation of B7.1 expression. In this study, we analyzed a B7.1 promoter element to identify the transcription factor binding sequences responsible for B7.1 gene transcription. We consistently observed a basal constitutive luciferase activity in cells transfected with the plasmid containing the B7.1 promoter linked to the luciferase reporter gene (pB7.1Pr.GL3E). The B7.1 promoter activity was inducible, because LPS stimulation of cells transfected with pB7.1Pr.GL3E resulted in a 3-fold increased luciferase activity compared with unstimulated cells. We identified a DNA sequence located between −84 to −72 bp upstream of the transcription start site and computer-aided analysis revealed that this binding element has similarities with the IRF-7-binding sequence. Mutation of the IRF-7 sequence in the B7.1 promoter construct abrogated the luciferase activity, suggesting the involvement of IRF-7 in the regulation of B7.1 gene transcription in LPS-stimulated monocyctic cells. Fong et al. (29) identified a B7-RE (~60 to ~47 bp upstream of the transcription start site) responsible for B7.1 gene transcription in EBV-transformed Raji B cells. This B7-RE activity was not inducible after stimulation and was not characterized. However, in our studies, transfection of THP-1/CD14 cells with the promoter construct containing sequences ~72 bp upstream of transcription start site containing B7-RE consistently failed to elicit luciferase activity in either unstimulated or LPS-stimulated cells. B7.1 expression has also been shown to be regulated in B cells by the NF-κB binding site located in the enhancer region ~3 kb upstream of the B7.1 transcription start site (28). Although we analyzed the
promoter region encompassing the sequences up to −587 bp relative to the transcription start site, the involvement of other transcription factors, particularly NF-κB, with binding sites located in the upstream enhancer region cannot be ruled out. IRF-7 has been shown to cooperate physically and functionally with coactivator proteins, including NF-κB, and CREB-binding protein/p300 (44, 45). These interactions not only stimulate the intrinsic IRF-7 transcriptional activity, but they are also indispensable for its ability to strongly synergize with other transcription factors, including c-Jun and IRF-3 (44–46). Whether IRF-7 mediates LPS-induced B7.1 expression by directly binding to the B7.1 promoter or through cooperative action with NF-κB needs to be understood.

The involvement of IRF-7 was also confirmed by competition studies in gel shift assays with an oligonucleotide probe corresponding to the consensus IRF-7 sequence represented by the PRD-1 oligonucleotides. The PRD-1 oligonucleotide sequences specifically bind the IRF-7 transcription factor (41). The findings that cold B7.1 oligonucleotides corresponding to the IRF-7 sequences inhibited the band detected after incubation of nuclear extracts with the PRD-1 probe suggested the involvement of IRF-7 in LPS-induced B7.1 transcription. However, we could not detect high m.w. complexes in our supershift experiments with anti-IRF-7 Abs (data not shown). This may be attributed in part to the unavailability of good commercial anti-IRF-7 Abs. Additionally, unidentified proteins present in the complexes formed after incubation of nuclear extracts with the oligonucleotide probes may have prevented the detection and formation of high m.w. complexes in supershift experiments. This was evident by the presence of nonspecific bands that could not be competed out with cold B7.1 oligonucleotides even when used at 400-fold higher concentrations than the labeled probe.

IRFs are a family of transcription factors comprising nine members with multiple functions, including apoptosis, oncosogenesis, host defense, viral latency, and immune responses (45, 46). The hallmark of this family of transcription factors is its N-terminal DNA-binding domain, which has well-spaced, conserved, five-trypotphan repeat sequences to form a helix-turn-helix motif that latches onto DNA (45, 47). The C-terminal region of IRF is variable and defines multiple biological functions (47). IRF-7, cloned and identified within the biological context of EBV latency, plays a critical role in the activation of IFN genes during viral infections to evoke antiviral responses and in the pathogenesis of some EBV-associated tumors (46, 48, 49). A number of agents, including LPS and viral infections such as Sendai and EBV, can induce the expression and activation of IRF-7 (46, 49). Although IRF-7 is a weak phosphorylation normally expressed in cytoplasm, augmentation of its phosphorylation by LPS/EBV-latent membrane protein-1 may facilitate localization of IRF-7 from the cytoplasm to the nucleus (47, 49, 50).

We have shown that LPS induces B7.1 expression through the activation of IRF-7 in human monocytic cells. The mechanism by which IRF-7 is activated and cooperates with other transcription factors is not fully elucidated, although an unknown virus-activated kinase has been suggested to phosphorylate IRF-7 (51). Very little is known about the signaling pathways involved in the regulation of B7 expression in general and particularly in response to stimulation of mononuclear cells with its most potent mitogen LPS. Very recently, PI3K was suggested to up-regulate B7 expression in EBV-transformed human B cells after ligation with CD40/CD40 homodimers (43). In this study we investigated the involvement of a number of signaling pathways, including calcium, PI3K, and

FIGURE 8. The IRF-7 binding site within the B7.1 promoter regulates LPS-induced B7.1 transcription in THP-1/CD14 cells. A, The positions of the potential regulatory elements in the B7.1 promoter region (−587 to +57 bp relative to the start site). The line diagram represents the extent of deletions within the B7.1 promoter region. Cells were cotransfected with 5 μg of either promoter deletion construct or vector control and with 2.5 μg of β-galactosidase control plasmid. After 24 h, cells were stimulated with LPS (1 μg/ml) for another 24 h. Luciferase activity was normalized for β-galactosidase activity to calculate the relative luciferase units (RLU). The results shown are the mean ± SD of six experiments performed in triplicate. B, The effect of mutating the IRF-7 sequence within the B7.1 −84 to −72 region on B7.1 promoter activity in LPS-stimulated THP-1/CD14 cells. A site-directed mutation of the B7.1 −84 to −72 sequence within the B7.1 promoter with underlined substituted nucleotides is shown in Fig. 7A. The amplified fragment (−84 to +57 bp) containing the mutations was cloned into a pGIL3E vector. Cells were cotransfected with either 5 μg of wild-type or mutant B7.1 constructs and with 2.5 μg of β-galactosidase control vector, followed by LPS stimulation and measurement of luciferase activities (RLU), as described above. Results shown are the mean ± SD of three independent experiments performed in triplicate.
MAPKs, in an attempt to elucidate the upstream signaling pathways involved in the activation of IRF-7 and the induction of B7.1 in LPS-stimulated monocytic cells. Our results clearly suggest that LPS-induced B7.1 expression involves the activation of JNK, but not of calcium, PI3K (data not shown), p38, or ERK MAPKs, in human monocytic cells. This conclusion was based on results from experiments using the JNK-specific inhibitor SP600125 and other agents, such as DXM and curcumin, which are known to inhibit the activation of JNK MAPKs (32). Studies designed to understand the signaling events downstream of JNK MAPK activation responsible for B7.1 gene transcription suggested that LPS-induced B7.1 expression may be regulated by IRF-7 through JNK activation. The IRF-7 activity, as determined by luciferase and gel shift assays, was completely inhibited by SP600125. It is not clear how JNK activates IRF-7; however, there is evidence to suggest that IRF-7 can be activated by JNK MAPK in response to DNA-damaging agents (52).

The JNK MAPK pathway includes JNK1, JNK2, and JNK3 (53). JNK1 and JNK2 are widely expressed in several tissues, whereas JNK3 is more selectively expressed in brain, testis, and heart (54). The JNK3 gene has been shown to be involved in neuronal cell death, whereas JNK1 and JNK2 have been implicated in T cell/human cell differentiation (55, 56). JNK1 has also been shown to regulate the development of T cell-mediated immunity against Leishmania major infections in an experimental mouse model (57). Our results show that down-regulation of JNK1 as well as JNK2 activation by their respective siRNAs resulted in a significant inhibition of LPS-induced B7.1 expression in THP-1 cells.

Although interaction of LPS with its receptor complex CD14/TLR4 results in the activation of several signaling cascades, including MAPKs, PI3K, and the calcium signaling pathways, and a host of transcription factors, it is surprising to observe that LPS-induced expression of B7.1 primarily involved the activation of JNK MAPKs and the IRF-7 transcription factor. B7.1 can also be induced by several other stimuli, such as IFN-γ on monocyte cells and CD40 stimulation on B cells (15, 18, 43). However, it remains to be determined whether IFN-γ or CD40-induced B7.1 expression involves the activation of JNK MAPK and/or the IRF-7 transcription factor. JNK MAPK has been suggested to play a key role in the regulation of immune responses, such as CD4⁺ Th cell differentiation and the production of macrophage-derived Th cytokines, including IL-12 (40, 58–60), and in several autoimmune diseases, including type I diabetes (61). Because B7 induction has also been suggested to play a key role in many such processes (4–12), it will be interesting to determine the overall involvement of JNK MAPK and IRF-7 in B7.1 induction and B7.1-mediated

![FIGURE 9. SP600125 inhibits LPS-mediated B7.1 promoter activity in THP-1/CD14 cells. Cells were cotransfected with 5 μg of either a promoter construct or a vector control and with 2.5 μg of β-galactosidase control plasmid. The transfected cells were untreated or pretreated with either SP600125 or calphostin C as a control for 2 h, followed by stimulation with LPS for 24 h and measurement of luciferase activity (relative luciferase units (RLU)) as described above. Results shown are the mean ± SD of three independent experiments performed in triplicate.](http://www.jimmunol.org/)

![FIGURE 10. JNK inhibitors inhibit IRF-7 binding to the B7.1 promoter in LPS-stimulated THP-1/CD14 cells. A. Cells were stimulated with LPS (1 μg/ml) for 0–5 h, followed by collection of cell pellets. Gel shift assays were performed on the cytoplasmic and nuclear extracts using the [32P]IRF-7 B7.1 sequence as a labeled probe. B. To determine the specificity of IRF-7 binding, the nuclear extracts were incubated with cold oligonucleotides (100- to 400-fold) corresponding to the sequence for IRF-7 B7.1 (left panel) or NF-κB (middle panel), followed by probing with the [32P]IRF-7 B7.1. The nuclear extracts were also incubated with cold IRF-7 B7.1 oligonucleotide (100- to 400-fold) for 20 min, followed by probing with the PRD-I32P-labeled oligonucleotides (right panel). C. THP-1/CD14 cells were treated with various concentrations of SP600125 or DXM for 2 h before stimulation with LPS (1 μg/ml) for 4 h. Nuclear extracts were incubated for 20 min with [32P]-labeled B7.1 oligonucleotide probe, and gel shift assays were performed. The complexes were subjected to gel shift electrophoresis, followed by autoradiography. NS, nonspecific.](http://www.jimmunol.org/)
effects using JNK- or IRF-7-null mice, respectively. If JNK knock-out mice are able to effectively block B7.1 induction in response to certain stimuli, such as LPS, and reduce the severity of some auto-immune diseases in experimental animal models, then JNK may represent an alternative pharmacological target for the design of a small molecule inhibitor that could modulate B7 induction and Th responses and therefore protect against autoimmune diseases.

In summary, to our knowledge this is the first report demonstrating the involvement of the IRF-7 transcription factor in the regulation of B7.1 gene transcription. Our results also suggest that JNK MAPK may play a critical role in IRF-7 activation and eventually the regulation of B7.1 expression in human monocytic cells in response to stimulation with LPS. IRF-7, a potent inducer of IFN-γ genes, appears to function in concert with other transcription factors, such as IRF-3, p300/CREB-binding protein, and c-Jun. Identification of factors that bind to IRF-7 in the context of B7.1 induction will facilitate understanding of the regulation of this important costimulatory molecule.

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Disclosures
The authors have no financial conflict of interest.

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