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Toll-like Receptor 2 and 4 (TLR2 and TLR4) Agonists Differentially Regulate Secretory Interleukin-1 Receptor Antagonist Gene Expression in Macrophages*

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Treatment of macrophages with lipopolysaccharide (LPS) from Gram-negative bacteria or peptidoglycan (PGN) from Gram-positive bacteria activates multiple intracellular signaling pathways and a large, diverse group of nuclear transcription factors. The signaling receptors for PGN and LPS are now known to be the Toll-like receptors 2 and 4 (TLR2 and -4, respectively). While a large body of literature indicates that the members of the TLR family activate nearly identical cytoplasmic signaling programs, several recent reports have suggested that the functional outcomes of signaling via TLR2 or TLR4 are not equivalent. In the current studies, we compared the responses of the secretory IL-1 receptor antagonist (sIL-1Ra) gene to both LPS and PGN. Both LPS and PGN induced IL-1Ra gene expression; however, the combination of both stimuli synergistically increased sIL-1Ra mRNA expression and promoter activity, suggesting that the signals induced by PGN and LPS are not equivalent. While both LPS and PGN utilized the PU.1-binding sites in the proximal sIL-1Ra promoter region to generate a full response, additional distinct promoter elements were utilized by LPS or PGN. Activation of p38 stress-activated protein kinase was required for LPS- or PGN-induced IL-1Ra gene expression, but the p38-responsive promoter elements localized to distinct regions of the sIL-1Ra gene. Additionally, while the LPS-induced, p38-dependent response was dependent upon PU.1 binding, the PGN-induced, p38 response was not. Collectively, these data indicated that while some of the intracellular signaling events by TLR2 and TLR4 agonists are similar, there are clearly distinct differences in the responses elicited by these two bacterial products.

The molecular mechanisms involved in the regulation of cytokine genes in macrophages and monocytes in response to stimulation with bacterial products has been a topic of intense interest. Treatment of monocytes and macrophages with lipopolysaccharide from Gram-negative bacteria or peptidoglycan from Gram-positive bacteria leads to the production of a vast array of cytokines and chemokines (1) and activates multiple intracellular signaling pathways including the extracellular signal-regulated kinase, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinase families (2). Likewise, a large and diverse group of nuclear transcription factors are also activated, including NF-κB, AP-1, PU.1, and interferon regulatory factors (2). In the past few years, our understanding of the events occurring following the interaction of microbes with cells of the innate immune response has expanded remarkably.

The Toll-like receptors (TLRs)[1] are an evolutionarily conserved family of cell surface molecules that participate in innate immune recognition of pathogen-associated molecular patterns (PAMPs) (3). PAMPs are generally unique, chemically diverse products with conserved motifs that are produced by microorganisms. PAMPs often have an essential role in the structure of bacteria and generally cannot be subtly modified as a result of mutation. Examples include LPS (specifically lipid A), peptidoglycan (PGN), lipoproteins, bacterial DNA, and bacterial flagella. At least nine different TLRs have been identified. In some cases, the bacterial ligand has also been identified. For example, TLR2 recognizes peptidoglycan (4) and mycobacterial lipoarabinomannan (5), TLR4 recognizes LPS from most Gram-negative species, TLR5 reacts with flagellin (6), and TLR9 is a receptor for bacterial CpG DNA (7).

The signaling events occurring downstream of the TLRs are rapidly being elucidated and appear to have many common features. In general, the cascade of events occurring following ligation of the different TLRs involves the activation of a common set of adapter proteins and protein kinases, the best characterized of which leads to the activation of NF-κB (reviewed in (8). Whereas a large body of literature indicates that the members of the TLR family activate a nearly identical intracytoplasmic signaling program, several recent reports have begun to suggest that the functional outcomes of signaling via TLR2 or TLR4 are not equivalent. As early as 1996, Dziarski *et al.* (9) demonstrated that stimulation of RAW 264.7 macrophages with LPS or PGN resulted in similar, but not identical activation of mitogen-activated protein kinases. More recently, Hirschfeld *et al.* demonstrated that LPS derived from *Porphyromonas gingivalis* (a TLR2 ligand) or *E. coli* (a TLR4 ligand) induced differential expression of a number of genes in murine macrophages (10). Likewise, Jones, *et al.* demonstrated that a secreted TLR2 agonist from culture filtrates of *Mycobacterium tuberculosis* and *Escherichia coli* LPS induced distinct patterns of cytokine production by RAW 264.7 macrophages (11).

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[1] The abbreviations used are: TLR, Toll-like receptor; PGN, peptidoglycan; IL, interleukin; IL-1Ra, IL-1 receptor antagonist; sIL-1Ra, secreted IL-1Ra; icIL-1Ra, intracellular IL-1Ra; LPS, lipopolysaccharide; PAMP, pathogen-associated molecular pattern; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SAPK, stress-activated protein kinase; RT, reverse transcription.

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Interleukin-1 is one of the most highly inflammatory cytokines produced by monocytes/macrophages in response to stimulation with LPS. The discovery of a naturally occurring IL-1 receptor antagonist (IL-1Ra) has suggested a means of modulating the IL-1-induced inflammatory response (12–14). IL-1Ra is structurally related to IL-1 (13–15) but specifically blocks the binding of IL-1α and IL-1β to cell surface receptors without itself activating target cells (16, 17). The term IL-1Ra actually refers to three closely related proteins. The first form to be described, secretory or sIL-1Ra, was cloned from IgG-stimulated human monocytes and encodes a protein of 177 amino acids, including a 25-amino acid hydrophobic leader sequence, which is subsequently cleaved, resulting in a secreted 152-amino acid mature protein (14). An alternative form of IL-1Ra, intracellular or icIL-1Ra, was cloned from an adherent monocyte cDNA library (18). This structural variant is created when an alternative first exon is spliced into an internal acceptor site in the first exon of the sIL-1Ra RNA within the region encoding for the secretory leader sequence. Thus icIL-1Ra is identical to the mature sIL-1Ra protein except for seven additional amino acids at the amino-terminal end, and icIL-1Ra lacks the hydrophobic leader sequence required for secretion. At the genomic level, distinct promoters separated by nearly 10 kb of DNA control the expression of sIL-1Ra and icIL-1Ra. Both forms of IL-1Ra are equally effective at inhibiting IL-1-induced cellular responses *in vitro*. However, given the strictly cell-associated nature of icIL-1Ra, its role in modulating extracellular inflammatory responses remains to be determined. A third, low molecular weight form of IL-1Ra, termed icIL-1RaII, is derived from an alternative translation initiation at the second ATG of either the sIL-1Ra or icIL-1Ra mRNA (19). The biological role of this form is unknown.

Previously, we have demonstrated that both isoforms of the human IL-1Ra genes are transcriptionally activated in macrophages in response to LPS. Our own studies on the regulation of the human secretory IL-1Ra promoter have demonstrated that NF-κB, CCAAT/enhancer-binding protein, STAT6, and most recently PU.1 and GABP are involved in regulating gene expression in macrophages (20, 21). Of these, PU.1 appears to be the most critical for the response of the sIL-1Ra gene to LPS. The response of the sIL-1Ra gene to PGN has not previously been evaluated. In the current studies, we have compared the responses of the sIL-1Ra gene to both LPS and PGN. Here we report that both LPS and PGN can induce sIL-1Ra gene expression; however, the combination of both stimuli synergistically up-regulated sIL-1Ra gene expression and promoter activity, suggesting that the signals induced by PGN and LPS are not equivalent. Both LPS and PGN utilized the PU.1-binding sites in the proximal promoter region to generate a full response; however, additional distinct promoter elements were utilized by LPS or PGN. We determined that the activation of p38 SAPK was an important component of the response elicited by LPS and PGN but that the p38-responsive promoter elements localized to distinct regions of the sIL-1Ra gene. Additionally, while the LPS-induced p38-dependent response was dependent upon PU.1 binding, the PGN-induced, p38 response was not. Collectively, these data indicated that while some of the intracellular signaling events induced by TLR2 and TLR4 agonists are similar, there are clearly distinct differences in the responses elicited by these two TLR ligands.

EXPERIMENTAL PROCEDURES

Reagents

LPS (*E. coli* serotype 055:B5) was obtained from Sigma. Prior to use, LPS was subjected to an additional purification procedure as described by Hirschfeld *et al.* (22). This procedure resulted in LPS that was free from contaminating endotoxin protein and is a specific TLR4 agonist. Peptidoglycan purified from *Staphyloccous aureus* was purchased from Fluka. The selective p38 inhibitor SB203580 was obtained from Alexis Pharmaceuticals (San Diego, CA).

Quantitative Real Time Reverse Transcription (RT)-PCR Analysis

Total RNA was purified using the Trizol reagent (Invitrogen). Briefly, RT of 0.5 μg of total cellular RNA was performed in a final volume of 20 μl containing 5× first strand buffer (Invitrogen), 1 mM concentration of each dNTP, 20 units of placental RNase inhibitor, 5 μM random hexamer, and 9 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen). After incubation at 37 °C for 45 min, the samples were heated for 5 min at 92 °C to end the reaction and stored at −20 °C until PCR use. 2 μl of cDNA was subjected to real time, quantitative PCR using the iCycler (Bio-Rad) with SYBR Green I (Molecular Probes, Inc., Eugene, OR) as a fluorescent reporter. sIL-1Ra and GAPDH cDNAs were amplified in separate reactions. Threshold cycle number was determined using the iCycler software, and levels of sIL-1Ra expression were normalized to GAPDH levels using the formula $2^{(Rt - Et)}$, where *Rt* represents the threshold cycle for the reference gene (GAPDH) and *Et* is the threshold cycle for the experimental gene (sIL-1Ra). Data are thus expressed as arbitrary units. Primer sequences were as follows: sIL-1Ra F, AAATCTGCTGGGGACCCTAC; sIL-1Ra R, TCCCAGATTCTGAAGGCTTG; GAPDH F, GTGTGAACGGATTTGGCCGT; GAPDH R, GAGGTCAATGAAGGGGTCGT.

DNA Constructs

Human TLR2, TLR4, and MD-2 Expression Plasmids—Human TLR2 and TLR4 cDNAs corresponding to the entire coding regions were generated by RT-PCR using primers corresponding to the published sequences and cloned into pcDNA3.1Zeo (Invitrogen). The human MD-2 expression construct was generated by RT-PCR and cloned into pEF6/myc-His (Invitrogen) to generate a molecule containing amino-terminal c-Myc and His_6 tags. Sequences of all clones were confirmed by automated sequencing.

IL-1Ra Genomic Clone Isolation—A gridded array of P1 clones that was approximately 2 times representative of the human genome was a kind gift of Drs. Fiona Watt and Hans Lehrach (Imperial Cancer Research Fund, London, UK). The library was screened by hybridization with a cDNA probe for exon 2 of human *IL1RN*, and clone ICRF700G13105 was identified. The clone contained ~80 kb of genomic DNA. DNA was isolated and digested with various rare cutters. *Bst*ZI was used to generate a fragment that contained the entire ILRN gene, as determined by hybridization with oligonucleotides derived from exon 1 and the 3′-end of exon 4. A 23-kb *Bst*ZI fragment was subcloned into a pUC9 derivative that had been modified to replace its linker with a *Not*I site containing a stuffer sequence.[2] The stuffer sequence was removed with *Not*I, and the cohesive *Bst*ZI fragments from the P1 clone were ligated. Recombinant plasmids were screened by hybridization. Sequencing of the 5′-end of the insert indicated that the entire *IL1RN* gene was present. This construct was termed pRNZ1.

IL-1Ra Promoter/Luciferase Reporter Constructs—A 7.1-kb sIL-1Ra promoter/luciferase reporter plasmid was constructed by cloning a 6.8-kb *Kpn*I genomic fragment derived from pRNZ1 into the unique *Kpn*I site at −294 of the human sIL-1Ra promoter in pA_3Luc (20). Promoter deletions from the 5′-end were created by standard subcloning techniques using convenient restriction enzyme sites or PCR. Site-directed mutants were initially generated in the 294-bp proximal promoter construct by recombinant PCR and verified by sequencing. A 3.1-kb genomic fragment, corresponding to sequences from approximately −3400 to −294, was subcloned into pBS-SK and then inserted into the *Kpn*I site as above to generate mutants in the context of the full-length 3.4-kb sIL-1Ra promoter.

The human CD14 expression vector was a gift of R. Ulevitch (Scripps Research Institute, La Jolla, CA), and dominant negative MKK3 and MKK6 plasmids were gifts of R. J. Davis (University of Massachusetts, Worcester, MA). NF-κBLuc was from CLONTECH. All plasmid DNAs were isolated by using endotoxin-free preparation kits from Qiagen (Valencia, CA) or Stratagene (La Jolla, CA).

Cell Culture and Transfection

The RAW 264.7 murine macrophage cell line and HEK293 cells were obtained from ATCC and maintained in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT). RAW 264.7 cells were cultured and transfected, and luciferase activities were measured as previously described (21). HEK293 cells were transfected in

[2] M. Nicklin, unpublished observations.

24-well plates using LipofectAMINE (Invitrogen). Each transfection contained 500 ng of NF-κBLuc, 50 ng of TLR2 or TLR4, 50 ng of pEF6-MD2, 100 ng of pRc/RSVCD14, 200 ng of pTK-renilla (Promega), and 4 μl of LipofectAMINE. Transfections were performed in triplicate, cultured for 40 h, and then stimulated as indicated for an additional 8 h. Luciferase activities were determined using the dual luciferase kit from Promega, and all activities were normalized to the activity of the co-transfected TK-renilla plasmid.

Western Blot Analysis

Antibodies were obtained as follows: p38 from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); phospho-p38 from New England Biolabs; and anti-FLAG (M2) from Sigma. For analysis of p38 phosphorylation, RAW 264.7 cells were seeded into six-well tissue culture plates. Cells were stimulated for the indicated time with LPS or peptidoglycan, washed one time with cold phosphate-buffered saline, and lysed *in situ* with 100 μl of SDS sample buffer. DNA was sheared by passage through a 25-gauge needle; lysates were boiled for 5 min and iced; and 10 μl was loaded onto a 12.5% SDS-polyacrylamide gel. Separated proteins were electroblotted unto nitrocellulose, and phosphorylated p38 was detected with a 1:1000 dilution of the antibody according to the manufacturer's recommendation. Detection was carried out using the ECL reagent from Amersham Biosciences. Blots were stripped of antibodies by washing in 62.5 mM Tris-HCl (pH 6.7), 100 mM 2-mercaptoethanol, 2% SDS for 50 min at 50 °C. The filters were then washed in Tris-buffered saline plus 0.05% Tween 20 and reprobed for total p38.

For detection of FLAG-tagged dominant negative MKK3 or MKK6, equal amounts of protein from cell lysates of RAW 264.7 cells transfected with expression vectors for the dominant negatives or control empty vector were immunoprecipitated using the M2 antibody, electrophoresed through a 10% SDS-polyacrylamide gel, blotted to nitrocellulose, and detected using the anti-FLAG antibody according to the manufacturer's recommendations.

RESULTS

Highly Purified LPS and Peptidoglycan Are TLR4- and TLR2-specific Agonists, Respectively—Hirschfeld *et al.* (22) have demonstrated that commercially available preparations of LPS may be contaminated with endotoxin-associated proteins and as such are not pure TLR4 ligands. In order to establish that the reagents used in the following studies are TLR2- and TLR4-specific agonists, we tested their abilities to activate an NF-κB reporter plasmid in HEK293 cells engineered to express either TLR2 or TLR4. We constructed human TLR2 and TLR4 expression plasmids by cloning the coding regions for the two genes, generated by RT-PCR from human monocyte mRNA, into the pcDNA3.1 eukaryotic expression plasmid (Invitrogen). To validate this system, the experiment shown in Fig. 1 was performed. HEK293 cells were transiently transfected with the NF-κB/luciferase reporter, Rc/RSV-CD14, and either pcDNA3-TLR2 or pcDNA3-TLR4. 40 h after transfection, cultures were stimulated for 8 h with the indicated ligands.

As shown in Fig. 1, unextracted, commercially prepared LPS and peptidoglycan but not highly purified LPS were able to activate NF-κB in TLR2-transfected cells. In contrast, NF-κB was activated in TLR4-transfected cells by purified or unpurified LPS but not by peptidoglycan. These results confirmed that HEK293 cells engineered to express either TLR2 or TLR4 responded appropriately when stimulated with TLR2-specific (peptidoglycan) or TLR4-specific (repurified *E. coli* LPS) ligands. The dual specificity of the commercially prepared LPS for TLR2 and TLR4 suggests contamination of the preparations with endotoxin-associated proteins. However, as predicted, re-extraction of the LPS according to the previously described method (22) eliminated signaling via TLR2. These results therefore confirmed that the LPS and PGN preparations used in the following studies are pure TLR4 and TLR2 agonists, respectively.

LPS and Peptidoglycan Induce IL-1Ra Gene Expression—In earlier studies, we and others have demonstrated that the human IL-1Ra gene is transcriptionally up-regulated in macrophages in response to LPS (20, 24–26). The response of the IL-1Ra gene to Gram-positive bacterial products has not previously been examined. In Fig. 2*A*, we assessed the ability of LPS, PGN, or the combination to induce sIL-1Ra mRNA in RAW 264.7 macrophages. Cultures were stimulated for 4 h with 1 μg/ml LPS and/or 10 μg/ml PGN, total RNA was purified, and sIL-1Ra mRNA expression was assessed by quantitative RT-PCR. As expected, treatment with LPS resulted in a robust enhancement of sIL-1Ra mRNA expression. Peptidoglycan at 10 μg/ml was somewhat less effective in inducing mRNA expression. Surprisingly, the combination treatment with LPS and PGN resulted in a very large increase in sIL-1Ra mRNA expression that was greater than either LPS or PGN alone. This response occurred despite using quantities of LPS and PGN that were previously determined to result in maximal IL-1Ra expression (data not shown).

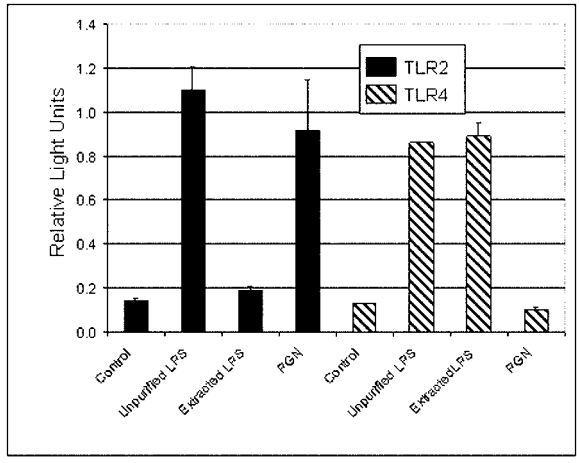

FIG. 1. **Highly purified LPS and peptidoglycan are TLR4- and TLR2-specific agonists, respectively.** HEK 293 cells were transiently transfected with NF-κBLuc, pRc/RSVCD14, pEF6MD2, and either pcDNA3.1-TLR2 or pcDNA3.1-TLR4 plasmids. Triplicate transfections were stimulated with 1 μg/ml LPS or 10 μg/ml PGN as indicated for 8 h prior to assay for luciferase activity as described under "Experimental Procedures." Data are from a single representative experiment of three performed.

Previously, we have demonstrated that the proximal 294 bp of the human sIL-1Ra promoter contained DNA elements required for the tissue-specific and LPS-inducible activity of the promoter (21). However, studies in which the β-galactosidase gene was placed under the control of a 1680-bp IL-1Ra promoter fragment and used to create a transgenic mouse demonstrated that, in fact, this region did not contain all promoter elements required for appropriate tissue- and stimulus-specific expression (27). In order to determine whether other, more distal, cis-acting DNA sequences were contained within the 5′-flanking region of the human sIL-1Ra gene, we generated luciferase reporter constructs containing up to ~7100 bp of sIL-1Ra DNA sequence upstream of the transcriptional start site. When transfected into RAW 264.7 cells, LPS induced approximately a 15-fold increase in promoter activity from the 7.1-kb promoter compared with 4-fold from the 294-bp promoter. Furthermore, deletions from the 5′-end of this region demonstrated that the minimal fully LPS- and PGN-responsive sIL-1Ra promoter consisted of 3400 bp of 5′-flanking sequence (data not shown).

In the experiment shown in Fig. 2*B*, this 3.4-kb sIL-1Ra promoter/luciferase reporter construct was transiently transfected in RAW 264.7 cells and the response to LPS, PGN, or the combination was assessed. Similar to what we observed for steady state mRNA expression, the human sIL-1Ra promoter

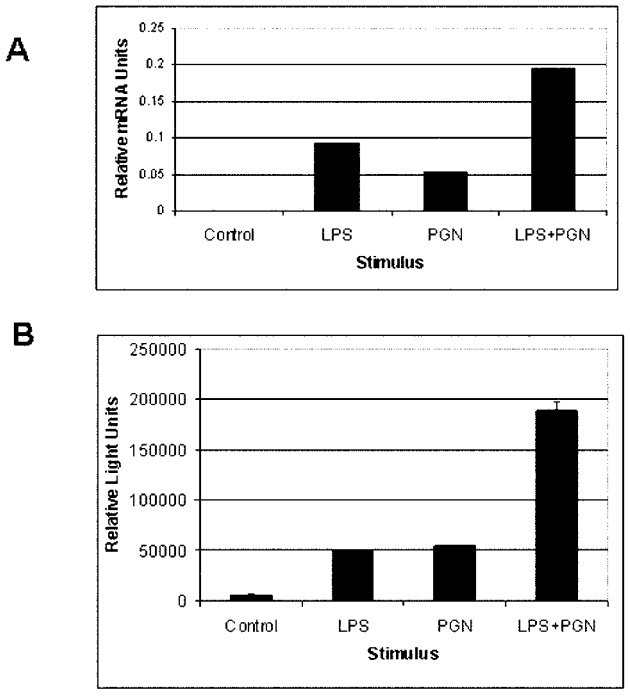

FIG. 2. **LPS and PGN induce sIL-1Ra gene expression.** *A*, quantitative RT-PCR analysis of sIL-1Ra gene expression in RAW 264.7 cells stimulated with 1 μg/ml LPS, 10 μg/ml PGFN, or the combination for 4 h prior to isolation of total cellular RNA. sIL-1Ra mRNA expression was normalized to GAPDH expression as described under "Experimental Procedures" and is expressed as arbitrary units. *B*, RAW 264.7 cells were transiently transfected with the 3.4-kb IL-1Ra/luciferase reporter and stimulated for 8 h with LPS, PGN, or the combination of both. Results indicate relative light units ± S.D. of triplicate transfections. One representative experiment of three performed is shown.

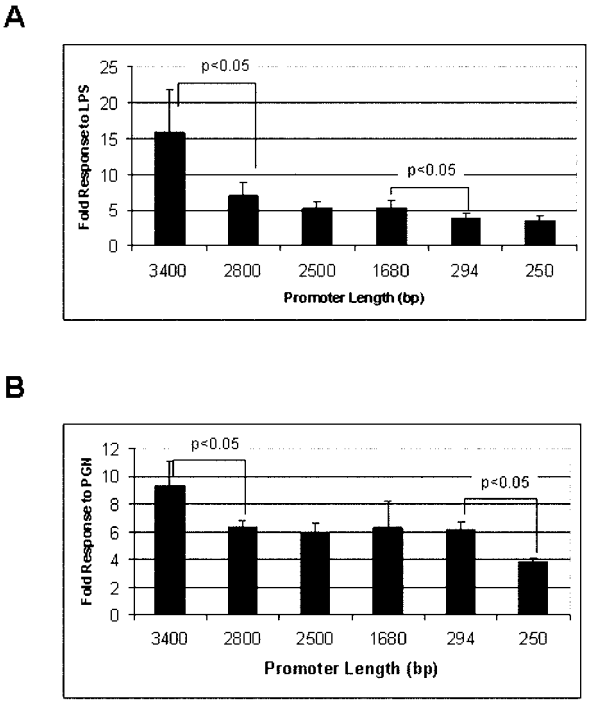

FIG. 3. **5′-Deletional mapping of LPS- and PGN-responsive promoter elements.** RAW 264.7 cells were transiently transfected with luciferase reporter constructs containing human sIL-1Ra promoter fragments of the indicated length. Cells were stimulated with either 1 μg/ml LPS (*A*) or 10 μg/ml PGN (*B*) for 8 h prior to harvest and assay for luciferase activity. Results indicate -fold response ± S.D. over unstimulated cells for each construct. $n \geq 4$ for each construct. p values are derived from Student's t test.

was activated approximately equally by LPS or PGN. The combination of LPS and PGN resulted in a synergistic 37-fold increase in promoter activity. This result suggested that distinct signals may be induced by PGN and LPS, which result in the activation of distinct transcription factors that regulate IL-1Ra promoter activity.

To determine whether the signals by PGN and LPS do indeed result in the regulation of IL-1Ra promoter activity through different cis-acting promoter elements, we assessed the responses of a series of 5′ promoter deletions to LPS or PGN. In the studies shown in Fig. 3, RAW 264.7 cells were transiently transfected with human sIL-1Ra promoter constructs ranging from 294-bp up to 3400 bp, and the activation of each was determined in response to stimulation of the cells with LPS or PGN. The results in Fig. 3*A* indicated the presence of two previously unidentified LPS-responsive promoter elements: one between −3.4 and −2.8 kb and a second between −1680 and −294 bp. Fig. 3*B* shows the results of experiments assessing the response of the same constructs to PGN. In this case, the PGN response also required sequences between −3.4 and −2.8 kb; however, there was no loss in response when the region between −1680 and −294 was deleted, suggesting that this region does not contain promoter sequences required for the response of the sIL-1Ra promoter to PGN. However, deletion of sequences between −294 and −250 resulted in a decrease in the response to PGN without a corresponding loss of LPS-induced promoter activity. Taken together, these results indicated that the response of the human sIL-1Ra gene to PGN or LPS is dependent on the activation of different groups of transcription factors, the implication of this result being that the treatment of cells with TLR2 or TLR4 ligands may result in the activation of different signal transduction programs.

Role of p38 SAPK in LPS- and PGN-induced IL-1Ra Gene Expression—LPS treatment of macrophages has been demonstrated to activate multiple mitogen-activated protein kinase family members including extracellular signal-regulated kinases 1 and 2, c-Jun N-terminal kinase, and p38 (reviewed in Ref. 28). In addition, Dziarski *et al.* (9) have demonstrated that LPS and PGN activate similar but not identical signal transduction pathways in macrophages. In particular, they indicated that p38 SAPK was strongly activated by LPS but only weakly activated by PGN. In the experiments shown in Fig. 4, we assessed the activation of p38 in RAW 264.7 cells treated with LPS and/or PGN using a phospho-p38-specific antibody, which only recognizes the tyrosine-phosphorylated and hence activated form of p38. Fig. 4*A* shows the results of an experiment in which the activation of p38 was assessed following a 10-min stimulation with LPS and/or PGN. Consistent with the results of Dziarski's study, we observed that LPS was a stronger activator of p38 than PGN. Additionally, treatment of cells with a combination of LPS and PGN resulted in a level of p38 phosphorylation that was equivalent to that observed with LPS alone. To more closely examine the activation of p38 SAPK following LPS or PGN stimulation, the time course experiment shown in Fig. 4*B* was performed. LPS induced a very rapid (<5 min) phosphorylation of p38 that was maximal by 15 min and decreased to a lower steady state level by 45 min. In contrast, the activation of p38 in response to PGN was reproducibly delayed by 10–15 min compared with LPS. Similar to LPS, PGN-activated p38 decreased to a low steady state level, although, like the induction phase of the response, it was also delayed compared with LPS. Notably, both LPS and PGN were capable of activating p38 to comparable levels albeit with different kinetics.

In the following studies, we assessed the role of p38 SAPK on the regulation of sIL-1Ra gene expression in response to PGN

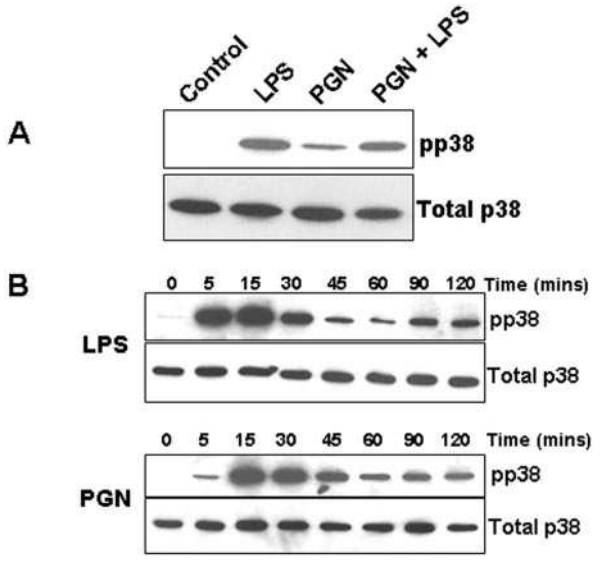

FIG. 4. **Activation of p38 SAPK by LPS and PGN.** RAW 264.7 cells were stimulated with LPS (1 μg/ml), PGN (10 μg/ml), or the combination of both for 10 min (*A*) or for the indicated time (*B*). Whole cell lysates were run on a 12% SDS-PAGE gel. Western analysis was performed using an antibody specific for the phosphorylated form of p38 (pp38). Blots were stripped and reprobed with an antibody that recognizes total p38 (*Total p38*). Immunoreactive bands were visualized by chemiluminescence.

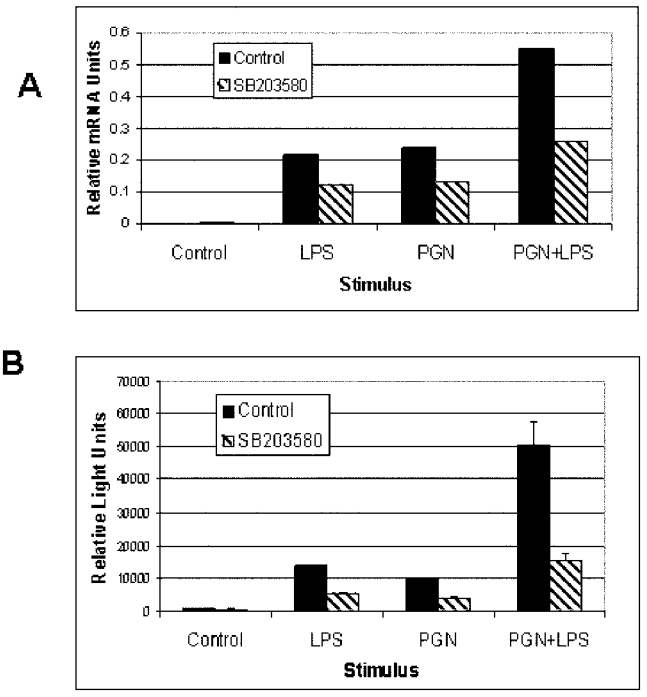

FIG. 5. **SB203580 inhibits LPS-, PGN-, and LPS plus PGN-induced sIL-1Ra gene expression.** *A*, quantitative RT-PCR analysis of sIL-1Ra mRNA expression from RAW 264.7 cells that were treated with 1 μg/ml LPS, 10 μg/ml PGN, or LPS plus PGN for 4 h with or without a 30-min pretreatment with 1 μM SB203580. sIL-1Ra mRNA expression was normalized to GAPDH expression as described under "Experimental Procedures" and is expressed as arbitrary units. *B*, RAW 264.7 cells were transiently transfected with the 3.4-kb sIL-1Ra promoter/luciferase reporter construct. Triplicate cultures were treated with LPS, PGN, or PGN plus LPS for 8 h with or without a 30-min pretreatment with 1 μM SB203580. Results are from one representative experiment of three performed.

and LPS. Cultures of RAW 264.7 cells were treated with a 1 μM concentration of the highly selective p38 inhibitor SB203580 for 30 min prior to stimulation with 1 μg/ml LPS, 10 μg/ml PGN, or the combination for 4 h. Total RNA was isolated, and sIL-1Ra mRNA accumulation was analyzed by quantitative RT-PCR. As shown in Fig. 5*A*, pretreatment with SB203580 inhibited the accumulation of LPS- or PGN-induced IL-1Ra mRNA. As demonstrated in Fig. 2, the combination of LPS plus PGN resulted in a synergistic induction of sIL-1Ra mRNA, which was also decreased by inhibition of p38. One possible explanation for this finding is that p38 SAPK affects the stability of the IL-1Ra mRNA. However, the IL-1Ra mRNA does not contain the typical AUUUA sequences found in other labile cytokine mRNAs that are regulated by p38. Thus, we examined the role of LPS- or PGN-activated p38 in the activation of the human sIL-1Ra promoter. In the transient transfection experiment shown in Fig. 5*B*, RAW 264.7 cells were transfected with the full-length 3.4-kb IL-1Ra promoter/luciferase reporter construct. 30 min prior to stimulation, cultures were treated with 1 μM SB203580 followed by stimulation with LPS and/or PGN for an additional 8 h. Consistent with the results from the Northern blot experiment, SB203580 was able to inhibit the activation of the sIL-1Ra promoter in response to LPS, PGN, or the combination of LPS and PGN. Taken together, these data indicate that both LPS and PGN regulate sIL-1Ra gene expression, in part, through the activation of p38 SAPK.

LPS- and PGN-activated p38 Regulate the sIL-1Ra Promoter through Different Cis-acting Elements—The studies shown in Fig. 3 indicated that LPS and PGN regulate sIL-1Ra gene expression through the use of different cis-acting promoter regions. In the following studies, we sought to determine whether the responses to LPS-activated and PGN-activated p38 SAPK map to the same regions within the sIL-1Ra promoter. RAW 264.7 cells were transiently transfected with the same series of promoter deletion constructs used in the experiments shown in Fig. 3, and the responses to LPS or PGN were determined in the presence or absence of 1 μM SB203580. As shown in Fig. 6*A*, the LPS-induced responses of all of the constructs, with the exception of the −294 promoter, were decreased as a result of inhibition of p38. This result therefore indicates that the region of the IL-1Ra promoter between −1680 and −294 contains a p38-responsive cis-acting element that probably binds transcription factors that are acted upon either directly or indirectly by p38. This region of DNA contains putative binding sites for several potential targets of p38 including CCAAT/enhancer-binding protein and AP-1. Interestingly, when cloned upstream of a minimal herpes simplex virus thymidine kinase promoter, the region between −1680 and −294 did not demonstrate any LPS-inducible activity, suggesting that other promoter elements are required for the ability of these sites to induce sIL-1Ra gene expression (data not shown).

In contrast, the PGN-induced responses of all of the constructs, except for the −250 bp promoter construct, were equally inhibited by SB203580 (Fig. 6*B*). This result suggests that the PGN-induced, p38-dependent cis-acting element is distinct from the LPS-induced element and lies between −294 and −250. These surprising results thus suggest that, although both LPS and PGN activate p38 SAPK, the functional outcomes of those events are distinct.

LPS and PGN Induce IL-1Ra Expression via MKK3 in RAW 264.7 Macrophages—In order to more closely examine the mechanism through which LPS and PGN induce IL-1Ra expression, we sought to determine the roles of MKK3 and MKK6 in activating p38 SAPK and subsequently the IL-1Ra gene. The following experiments were also undertaken to confirm that the results with SB203580 were in fact due to its ability to inhibit p38 and not to due other potential effects such as inhibition of protein kinase B as previously described (29). To inhibit activation specifically via MKK3 or MKK6, RAW 264.7 cells were cotransfected with either the 3.4-kb or 294-bp sIL-

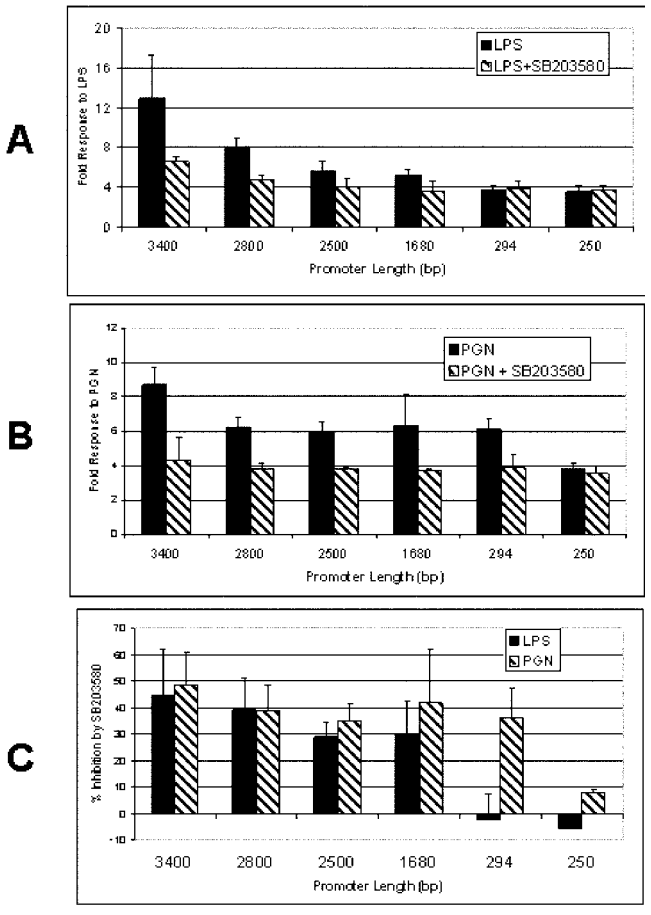

FIG. 6. **LPS- and PGN-activated p38 regulate the sIL-1Ra promoter through different cis-acting elements.** RAW 264.7 cells were transiently transfected with the indicated IL-1Ra promoter/luciferase reporter construct and stimulated with 1 μg/ml LPS or 10 μg/ml PGN in the presence or absence of 1 μM SB203580 for 8 h. *A*, -fold response to LPS ± S.D. of at least four transfections with each construct. *B*, -fold response to PGN ± S.D. of at least four transfections with each construct. *C*, percent inhibition of the response to LPS or PGN by pretreatment with SB203580. Data represent means ± S.D. from at least four experiments with each construct.

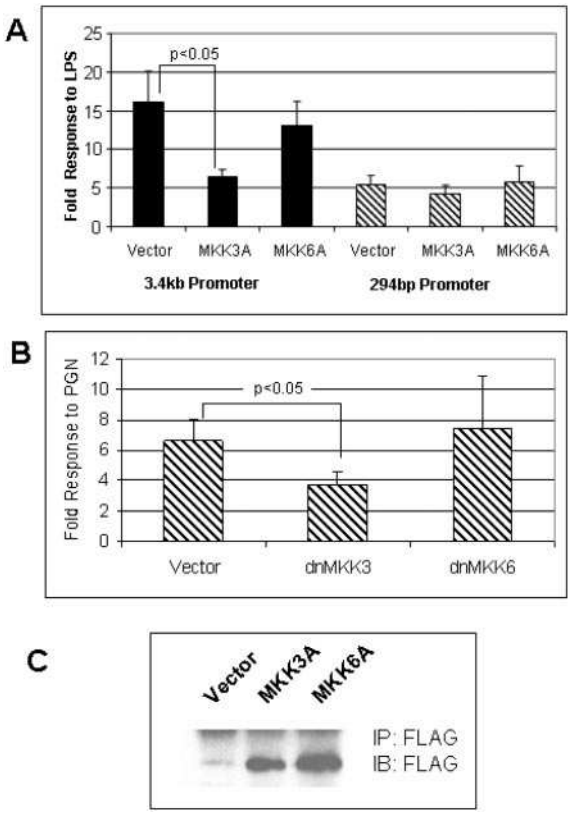

FIG. 7. **LPS and PGN induce sIL-1Ra promoter activity via MKK3.** RAW 264.7 cells were transiently transfected with sIL-1Ra promoter/luciferase reporters and expression vectors for dominant negative MKK3, MKK6, or empty vector. Cultures were stimulated with 1 μg/ml LPS or 10 μg/ml PGN for 8 h prior to assay for luciferase activity. Results are means ± S.D. of three separate experiments. *p* values were determined by paired *t* test. *A*, LPS response. Cells were transfected with either the 3400- or 294-bp IL-1Ra promoters. *B*, PGN response. Cells were transfected with the 294-bp IL-1Ra promoter reporter. *C*, immunoblot of whole cell lysates from transfected cells demonstrating the expression of the FLAG-tagged dominant negative MKK proteins as described under "Experimental Procedures."

1Ra promoter/luciferase reporter constructs and dominant negative MKK3 or MKK6 expression plasmids. As shown in Fig. 7*A*, LPS-induced IL-1Ra promoter activity was inhibited by cotransfection of dominant negative MKK3 but not MKK6. This inhibition was equal to that observed when the cells were treated with SB203580. Consistent with the experiments shown in Fig. 6, the LPS-induced activity of the 294-bp promoter was not inhibited by dominant negative MKK3 or MKK6. Likewise, in Fig. 7*B* we examined the effect of dnMKK3 or dnMKK6 on PGN-induced activity of the 294-bp IL-1Ra promoter. Again, consistent with results shown in Fig. 6, dnMKK3 but not dnMKK6 inhibited PGN-induced sIL-1Ra promoter activity. Immunoprecipitates of lysates from transfected cells using an anti-FLAG antibody demonstrated that the epitope-tagged MKK3 and MKK6 proteins were in fact both expressed (Fig. 7*C*).

Role of PU.1-binding Sites in LPS- and PGN-induced Gene Expression—The proximal 294-bp sIL-1Ra promoter contains two PU.1-binding sites that we previously demonstrated to be critical for the response of the proximal promoter to LPS (21). One of these sites, located at −81 to −93, is a composite NF-κB/PU.1/GABP binding site. Our earlier studies demonstrated that GABP did not participate in the LPS response of the IL-1Ra promoter; however, NF-κB and PU.1 did. To assess the role of PU.1 in regulating the activity of the full-length sIL-1Ra promoter in response to LPS- and PGN-activated p38, a series of site-directed mutants at the two proximal PU.1-binding sites were generated in the context of the 3.4-kb promoter fragment.

As shown in Fig. 8*A*, mutation of the downstream PU.1/NF-κB site (Fig. 8*B*) resulted in a loss of LPS responsiveness that could be further inhibited by SB203580. Likewise, mutation of the upstream PU.1 site located at −225 (*C*) resulted in an ~40% decrease in the LPS response, consistent with our previously published studies and was significantly inhibited by treatment with SB203580. However, mutation of both sites (*D*) resulted in a further loss of LPS responsiveness that was not affected by inhibition of p38. Since the LPS responsive element between −81 and −93 is a composite NF-κB/PU.1-binding site, we wanted to clarify the role of PU.1 or NF-κB in regulating p38-responsive IL-1Ra promoter activity. Site-directed mutants, in the context of the full-length 3400-bp promoter, were generated, which contained a mutation within the −225 PU.1 site as well as specifically blocked PU.1 or NF-κB binding to the −81 to −93 site (21). Mutation of the PU.1 half-site (*E*) resulted in a promoter construct with the same functional characteristics as the double mutant: low response to LPS and no inhibition by SB203580. However, mutation of the NF-κB half-site (*F*) resulted in a promoter construct with increased LPS responsiveness that was also inhibited by treatment with SB203580. Taken together, these results demonstrated that, in

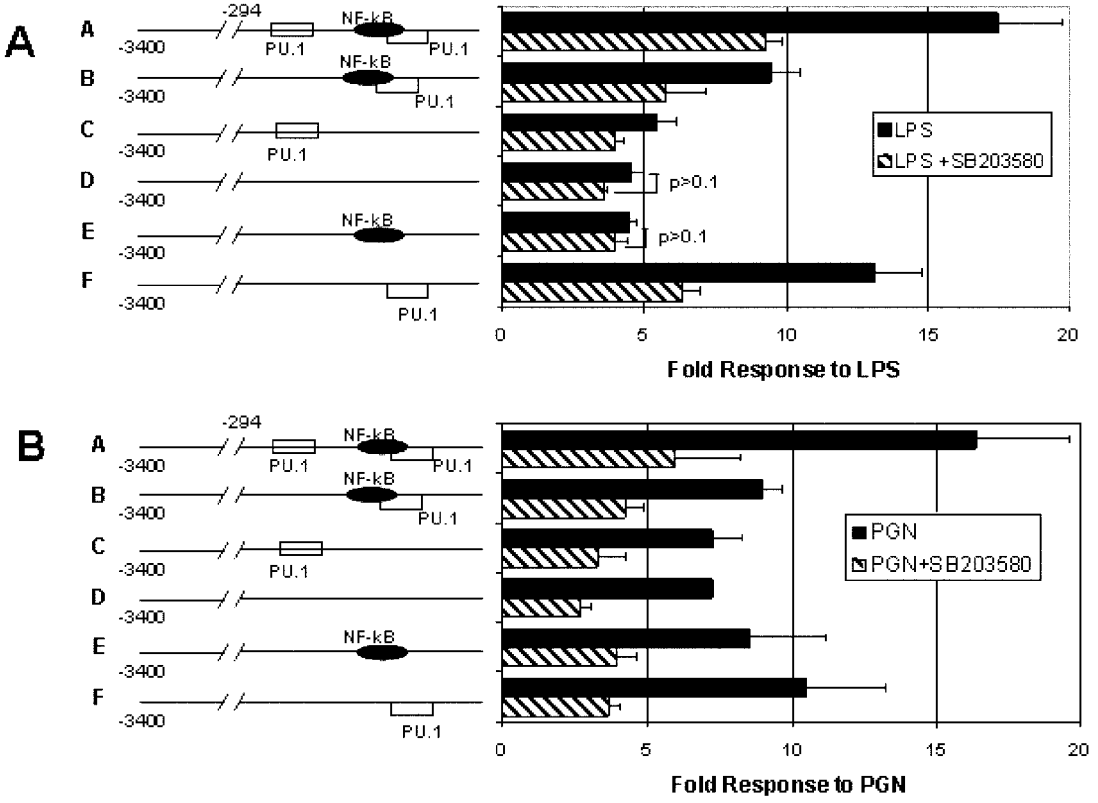

FIG. 8. **Role of PU.1-binding sites in LPS- or PGN-induced sIL-1Ra promoter activity.** RAW 264.7 cells were transfected with the indicated site-directed mutant sIL-1Ra promoter/luciferase reporter. Cultures were stimulated with 1 μg/ml LPS or 10 μg/ml PGN in the presence or absence of 1 μM SB203580 as described in Fig. 6. Results represent means ± S.D. of at least three separate experiments with each construct. p values were determined by paired t test. *A*, LPS response. Responses that were not significantly inhibited by SB203580 are indicated. *B*, PGN response. The responses of all constructs were significantly inhibited by SB203580 ($p < 0.05$).

the context of the full-length 3.4-kb sIL-1Ra promoter, PU.1 binding is critical for full responsiveness to LPS. Additionally, the p38-dependent promoter activity also requires PU.1. However, the ability of PU.1 alone to regulate LPS-inducible expression of the sIL-1Ra gene does not appear to be directly dependent on p38, since the LPS response of the intact proximal 294-bp promoter, which contains both PU.1-binding sites, was unaffected by treatment with SB203580 (Fig. 6, *A* and *C*).

The role of the proximal PU.1 sites in the control of PGN-induced promoter activity was also examined (Fig. 8*B*). The response of the sIL-1Ra promoter to PGN was also dependent upon the presence of the PU.1 sites; however, this dependence was clearly less than that of LPS as evidenced when both PU.1 sites are mutated (Fig. 8*B*, *lanes D* and *E*). Mutation of both PU.1 sites reduced the response of the promoter to LPS by ~75%, but the PGN response was only reduced ~50%. More striking was the lack of a requirement for PU.1 in response of the sIL-1Ra gene to PGN-activated p38. Unlike LPS, which had an absolute requirement for at least one PU.1-binding site in order to respond via p38, mutation of the PU.1 sites had no effect on the ability of SB203580 to inhibit PGN-induced promoter activity. Collectively, these results indicated that 1) PU.1 is not likely to be a direct target of p38 action; 2) the transcription factor activated by LPS-induced p38 may require the presence of PU.1 for its function; and 3) the PGN-induced, p38-responsive factor works independently of PU.1

DISCUSSION

Over the past few years, our understanding of the molecular mechanisms involved in the response of cells of the innate immune system to microbial products has increased dramatically. The identification of mammalian Toll-like receptors as the cell surface proteins that distinguish between different bacterial products and transduce signals to the responding cell was a highly significant finding. Interestingly, although the different TLRs recognize a wide variety of chemically diverse bacterial products, most studies have indicated that they activate a similar series of intracellular signaling molecules (reviewed in Ref. 30). Several recent papers have suggested that the signals generated by TLR2 and TLR4 are not equivalent. Hirschfeld *et al.* (10) compared the responses of 11 different genes in macrophages to LPS derived from *E. coli* or *P. gingivalis* (TLR4 and TLR2 ligands, respectively). While the induction of mRNA for genes such as macrophage inflammatory protein-1α and IL-1β was equivalent for *E. coli* or *P. gingivalis* LPS, other genes such a IL-6 and IL-12p40 were at best only weakly induced by the TLR2-specific LPS. Likewise, Jones *et al.* (11) demonstrated that whereas both LPS and a soluble TLR2 agonist from culture filtrates of *M. tuberculosis* (mannosylated phophatidylinositol) could induce tumor necrosis factor-α production, only LPS was capable of inducing IL-1β and nitric oxide secretion. One possible molecular mechanism for the differential signaling downstream of TLR2 and TLR4 has been provided by the recent identification of a second, receptor-proximal adapter protein (in addition to MyD88) that participates in TLR4 but not TLR2 signaling (31, 32).

In this report, we have provided evidence that different Toll-like receptor ligands can activate the same gene through different mechanisms. We studied the regulation of the gene for the secreted IL-1 receptor antagonist in response to the TLR2 agonist PGN and the TLR4 agonist *E. coli* LPS. While both PGN and LPS could induce sIL-1Ra gene expression and promoter activity to approximately equal levels, the combination of the two stimuli resulted in a synergistic increase in both mRNA accumulation and promoter activity. This result sug-

gested that stimulation of macrophages with PGN or LPS did not generate equivalent intracellular signals. These findings were confirmed when we determined that different regions of the human sIL-1Ra promoter were required for full responsiveness to LPS or PGN. Whereas the responses to both LPS and PGN required the previously identified PU.1-binding sites in the proximal promoter region, the response to LPS also utilized at least two more distally located promoter elements. The response to PGN required an additional element within the proximal 300-bp and an additional region located between 3400 and 2800 bp upstream of the transcription start site. The most distal response element for PGN co-localized with a region required for full LPS-responsiveness. Although we have not yet identified the transcription factors that are differentially activated by LPS and PGN, these are the first studies that we know of that clearly indicate that activation of macrophages via LPS and PGN can result in the activation of different DNA-binding proteins. Studies are currently in progress to definitively identify the transcription factors that are differentially activated in response to LPS and PGN.

In these studies, we also examined the role of p38 SAPK in the regulation of IL-1Ra gene expression induced by PGN and LPS. Treatment of macrophages with either LPS or PGN resulted in the activation of p38 SAPK, albeit with somewhat different kinetics (Fig. 4). Our results are generally consistent with those previously published by Dziarski *et al.* (9), who demonstrated that LPS was a more potent inducer of p38 activity than PGN. The reason for the delayed activation of p38 in response to PGN is unclear but could relate to receptor density or the requirement for other TLRs, such as TLR6, to be recruited into the complex (33).

Northern blot analysis of LPS and/or PGN treated RAW 264.7 cells demonstrated that pretreatment with the p38-inhibitory pyridinyl imidazole compound SB203580 resulted in a decrease in IL-1Ra mRNA expression. The mode of action of p38 in the regulation of LPS-induced gene expression is very much gene-dependent. In some instances (*e.g.* tumor necrosis factor-α, IL-6, and macrophage inflammatory protein-1α), inhibition of p38 activity was demonstrated to result in accelerated mRNA decay (34). However, inhibition of p38 activity blocked LPS-induced neuroleukin and interferon-stimulated gene 15 gene expressions without affecting mRNA stability, suggesting a role for p38 in the transcriptional regulation of those genes (35). The ability of p38 to regulate mRNA stability is likely to relate to the presence of AU-rich elements found within the 3′-untranslated regions of many cytokine genes (34, 36, 37). The sequence of the IL-1Ra 3′-untranslated region does not contain the typical AUUUA sequences found in other labile cytokine mRNAs that are regulated by p38 and is unlikely to be regulated by p38-dependent post-transcriptional mechanisms. This hypothesis was confirmed in transient transfection assays with IL-1Ra promoter constructs. Similar to the mRNA analysis, pretreatment of RAW 264.7 cells with SB203580 prior to LPS and/or PGN stimulation significantly inhibited promoter activity. Thus, both TLR2-induced and TLR4-induced IL-1Ra expression have as a requirement the activation of p38 SAPK. Surprisingly, the LPS- and PGN-induced p38 responses mapped to different regions of the IL-1Ra promoter and displayed differing requirements for the previously identified PU.1-binding sites.

The mechanism through which PU.1 regulates LPS-inducible gene expression is unclear. Earlier studies determined that PU.1 can be inducibly phosphorylated by casein kinase II in response to LPS stimulation of macrophages, and this phosphorylation appears to be required for the transactivation function of PU.1 (38). In the studies presented here, the LPS-responsive 294-bp IL-1Ra promoter, which contains two PU.1-binding sites, was not sensitive to inhibition of p38 SAPK, suggesting that PU.1 was not a target of the p38 signaling cascade. However, the data presented in Fig. 8 indicate that PU.1 is critical for the ability of the IL-1Ra gene to respond to LPS-induced p38 SAPK. These data would suggest that a functional interaction of PU.1 with transcription factors that bind to the distal promoter is critically dependent on the activity of p38 SAPK. Such a result would be consistent with the notion that a major role for PU.1 is to recruit other factors into the transcription complex (39–44). Additionally, these are the first data indicating a role for PU.1 in the regulation of gene expression induced by PGN.

The p38 SAPK family contains four distinct isoforms, p38α, p38β, p38γ, and p38δ; however, only p38α and p38β are sensitive to inhibition by SB203580 (45). The activities of the p38 family members are regulated by phosphorylation of a conserved TGY motif in kinase subdomain VIII by MKK6 or, with the exception of p38β, by MKK3 (46). Our studies using SB203580 to inhibit p38 activity therefore indicated that LPS- or PGN-induced sIL-1Ra gene expression was regulated by either p38α or p38β. In the experiments shown in Fig. 7, we demonstrated that only MKK3 was involved in the regulation of IL-1Ra promoter activity. Since MKK3 cannot activate p38β, these results indicate that LPS- or PGN-induced IL-1Ra gene expression is controlled, at least in part, via the MKK3-dependent activation of p38α.

A major question to arise from these studies is: How is it that, although both LPS and PGN share a common group of signaling intermediates and indeed are both capable of activating p38 SAPK, the two stimuli appear to activate different transcription factors? Given the large number of known TLRs that respond to a wide variety of ligands and the apparent similarities in the signaling mechanisms thus far identified for them, it would seem as if a mechanism would need to be in place in order for an organism to generate a response appropriate for and specific to a given pathogen. At least two different mechanisms can be envisioned for this.

One possible explanation is that the TLR2 and TLR4 signaling complexes are ultimately linked to different transcription factors through as yet unidentified scaffolding proteins. Recently, the role of scaffolding proteins in the control of the specificity of activation and function of the mitogen-activated protein kinase modules has been a topic of keen interest (reviewed in Refs. 23, 47, and 48). Although a clear demonstration of a transcription factor being included in a "signalsome" complex has not as yet been provided, such a mechanism cannot as yet be excluded. Having the different TLRs linked to specific transcription factors through scaffolding proteins could provide a mechanism to generate such specificity.

A second and perhaps more plausible explanation may be that although both receptors activate p38, each activates a different second signal, and it is the second signal that determines where the p38 response maps to. The studies reported herein and in the recent literature (10, 11, 31, 32) clearly indicate that the signals transmitted via different TLRs are not equivalent. Thus far, a significant amount of work has focused on signaling mechanisms that the different TLRs have in common (*e.g.* NF-κB). These recent studies now indicate that we need to look more closely at the differences.

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