Microbial Products Stimulate Human Toll-like Receptor 2 Expression through Histone Modification Surrounding a Proximal NF-κB-binding Site*

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Previous studies have yielded conflicting results regarding the ability of microbial products to activate TLR2 gene expression in human monocytes. In this study, we found that TLR2 mRNA was rapidly up-regulated in human monocytes treated with TLR2 and TLR4 agonists, and this corresponded to an increase in cell surface receptor levels. This induction was abrogated by actinomycin D as well as a pharmacologic inhibitor of transcription initiation site of the TLR2 gene, and sequence examination revealed a near-consensus NF-κB-binding element immediately upstream of this site. Electromobility shift assays confirmed that NF-κB bound to this putative site in vitro. However, luciferase reporter plasmids driven by the TLR2 promoter were not responsive to TLR2 agonists. Overexpression of the NF-κB p65 subunit was sufficient to induce expression of the endogenous TLR2 mRNA, and co-transfection of the CREB-binding protein and p300 co-activators further increased TLR2 mRNA levels. Chromatin immunoprecipitation analysis revealed that p65, CREB-binding protein, and p300 are recruited to the TLR2 promoter upon stimulation of human monocytes followed by histone hyperacetylation. Taken together, these results define a mechanism whereby histone modification and increased promoter access induce expression of human TLR2 following infection.

The recently discovered Toll-like receptor (TLR) family constitutes an essential component of the innate immune response to invading pathogens. Through direct recognition of conserved microbial products, TLRs enable the host immune response to sense the presence of microbes and initiate appropriate protective responses (1). Upon ligation, TLRs initiate cytoplasmic signaling pathways leading to the activation of transcription factors that induce pro-inflammatory mediators. Examples of TLR agonists include lipopolysaccharide (LPS) from Gram-negative bacteria (TLR4), bacterial flagellin (TLR5), unmethylated CpG-rich bacterial DNA (TLR9), and double-stranded viral RNA (TLR3) (1).

However, among all vertebrate TLRs, the most versatile with respect to ligand recognition is TLR2, which, in cooperation with either TLR1 or TLR6, recognizes a broad range of structures from bacteria, fungi, mycoplasma, and eukaryotic parasites (1). For example, TLR2 recognizes triacylated bacterial lipoproteins by forming a heterodimer with TLR1, and the TLR2/6 heterodimer recognizes mycoplasmal diacylated lipopeptides (2). Given the wide variety of pathogenic microbes that are recognized by TLR2, it is not surprising that this receptor (as well as its co-receptors) has been associated with susceptibility to various infectious diseases. Studies in mice have linked Tlr2 deficiency with vulnerability to infection with Staphylococcus aureus, Listeria monocytogenes, Legionella pneumophila, Mycobacterium tuberculosis, and others (3). In addition, genetic association studies of human TLR polymorphisms have confirmed the role of TLR2 in protection from infectious disease (4).

Consequently, many aspects of TLR2 structure and function have become the subject of investigation in recent years. Among these is the regulation of TLR2 expression. Given the established importance of TLR2 in various infectious diseases, as well as the potential for inappropriate activation of TLR2 signaling leading to chronic inflammation, there are likely to be mechanisms in place that tightly control expression of TLR2. TLR2 is expressed predominantly in immune cells of myeloid origin such as monocytes, macrophages, and dendritic cells (5–7). Furthermore, many studies have established that TLR2 is up-regulated in blood cells taken from patients suffering from various inflammatory disorders such as bacterial sepsis, underscoring the importance of TLR2 regulation in human disease (8–16). Several groups have begun to characterize the response of the TLR2 gene to various pro-inflammatory stimuli. Murine Tlr2 has been shown to be induced by whole bacteria as well as LPS and inflammatory cytokines (17). In addition, the murine Tlr2 promoter has been analyzed and found to contain binding sites for NF-κB and Sp1, both of which are required for transcriptional induction (18–20).
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Studies of human TLR2 regulation have, however, yielded inconsistent results, with some groups showing that the gene is up-regulated at the mRNA level in stimulated cells (5, 21) and others showing the opposite result (7, 22). We believe that these inconsistencies can be attributed to variations in experimental design among these studies. Therefore, in this study, we carefully reexamine the response of the human TLR2 gene to various microbial stimuli. We report that in primary monocytes stimulated with bacterial TLR ligands, TLR2 is rapidly up-regulated at both the messenger RNA and cell surface protein levels, and we offer an explanation for previous discrepancies on this issue thus reconciling the seemingly contradictory published data. We further show that NF-κB binds to the TLR2 promoter and is required for induction, suggesting that although the mouse and human TLR2 promoters share little sequence homology, they are functionally similar. Finally, we characterize a novel mechanism for human TLR2 transcriptional activation involving NF-κB, the transcriptional co-activators CBP and p300, and histone modification. These results suggest that up-regulation of TLR2 in response to microbial challenge may be one mechanism by which the immune system shapes the inflammatory response following infection.

EXPERIMENTAL PROCEDURES

Reagents—Synthetic bacterial lipopeptides, N-palmitoyl-S-[2,3-bis(palmitoyloxy)-propyl]-(R)-cysteinyl-(lysyl)-lysine (Pam3CSK4), and S-[2,3-bis(palmitoyloxy)-propyl]-(R)-cysteinyl-GNNDESNI-FKEK (macrophage-activating lipopeptide-2 (MALP-2)) were purchased from EMC Microcollections (Tuebingen, Germany). Repurified Salmonella minnesota Re595 lipopolysaccharide was purchased from List Biologicals (Campbell, CA). Actinomycin D and ammonium pyrrolidinedithiocarbamate (PDTC) were purchased from Sigma. Anacardic acid was purchased from Calbiochem.

Plasmids—the TLR2 promoter −2800 luciferase reporter plasmid was constructed as follows. Approximately 2.8 kb of promoter region, including the first noncoding exon of TLR2, was amplified from a bacterial artificial chromosome (Library RP11, clone 153M7, Invitrogen) using the following primers: forward 5′-AGCTAGATCTCGGACATACGACATCTGTCGACGAG-3′ and reverse 5′-AGCTAAGCTTTGGGAGAACTTCCGAGCAGTCA-3′. The PCR product was then cloned as a HindIII/KpnI fragment into the pFLAG-CMV-1 vector (Sigma). The CBP and p300 expression vectors encoding full-length, C-terminally hemagglutinin-tagged proteins were a generous gift from Dr. Richard Eckner (University of Medicine and Dentistry of New Jersey) (23). All plasmids were purified using the endo-free plasmid purification kit from Qiagen (Valencia, CA).

Cells and Transfections—Blood was obtained from healthy donors with informed consent under the University of Illinois Institutional Review Board approval. Primary monocytes were purified using the monocyte isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) following centrifugation through a Ficoll gradient. Monocyte preparations were >95% pure as determined by CD14 staining. Monocytes were cultured in RPMI 1640 medium supplemented with 10% autologous plasma and 1% l-glutamine. Unless indicated otherwise, monocytes in all experiments were cultured for 24 h following isolation. Primary monocytes and THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% l-glutamine. THP-1 and SW-620 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% l-glutamine. Plasmids were introduced into primary monocytes and THP-1 cells by electroporation using the Amaxa Nucleofector II (Amaxa, Cologne, Germany) according to the manufacturer’s instructions. 5 μg of DNA and 5 × 106 cells were used per condition. 293T cells were transfected using a CaCl2 method as described elsewhere (24) using 0.5 μg of each expression plasmid and 1.5 μg of empty vector per well in 6-well plates. SW-620 cells were transfected using FuGENE 6 (Roche Applied Science) as described previously (25). Luciferase assays were performed using the dual luciferase assay kit (Promega, Madison, WI).

Ribonuclease Protection Assay—The Ribonucent ribonuclease protection assay kit (BD Biosciences) was used according to the manufacturer’s instructions. 1 μg of total RNA and the human TLR-1 probe set were used. Protected fragments were resolved on a 4.75% polyacrylamide gel and detected using a Storm PhosphorImager (GE Healthcare). Relative RNA levels were quantified using ImageQuant software, and normalized to the housekeeping genes L32 and GAPDH.

Real Time PCR and Real Time RT-PCR—Real time PCR was performed using the SYBR green master mix and the Taqman ABI 7900 thermocycler from Applied Biosystems (Foster City, CA). For RT-PCR experiments, 1 μg of total RNA was reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen) prior to PCR amplification. Real time RT-PCR primer sequences were as follows: TLR1, forward 5′-ACAGAATTGTTGACGGATGTGTTTC-3′, reverse 5′-TTTGGATGGGCAAAGCATTGGAGAC-3′; TLR2, forward 5′-CAGGGGCTGTCTCGGAGGCTC-3′, reverse 5′-CACACACTCCAGTTGGAAGG-3′; and TLR6, forward 5′-CAAGTCTTCGGAAGGAAAGGACAC-3′, reverse 5′-GGTGCTGGACTTGTTTCTGC-3′, and GAPDH, forward 5′-GGTGAATTCACAGAATGACGAC-3′, and reverse 5′-TCGGAGAATTGTCTGCACT-3′. Final quantifications were performed using the ΔCqrt method (26) with GAPDH being used for normalization. Reactions performed in the absence of reverse tran-
scriptase were performed to confirm the specificity of the reactions for RNA.

5’ Rapid Amplification of cDNA Ends (5’ RACE)—Transcription start sites were mapped using the RNA ligase-mediated RACE kit from Ambion (Austin, TX). Briefly, an RNA adapter oligonucleotide (5’ RACE adapter) was ligated to the 5’ end of 1 µg of total RNA. This ligated RNA was then used as template for reverse transcription followed by nested PCR amplification. Gene-specific primer sequences used for endogenous TLR2 mRNA were as follows: outer 5’-GGTGATGCTCTGGTGTGAAGGTCAAG-3’ and inner 5’-GGTTCCCATTCCAGGCGATAATGG-3’. Gene-specific primers used for the TLR2 promoter luciferase reporter plasmid were as follows: outer 5’-GCGCAACTCGAATCCGATATATAACG-3’ and inner 5’-CATAGCTTCTGGCAAACCGGACC-3’.

PCR products were resolved on a 2% agarose gel and visualized with ethidium bromide. PCR products were subcloned using the Topo-TA cloning kit (Invitrogen), and at least 12 clones for each condition were sequenced.

Electromobility Shift Assay (EMSA)—EMSAs were performed as described elsewhere (27) with modifications. Briefly, 1 × 10⁷ monocytes were harvested and resuspended in sucrose lysis buffer (320 mM sucrose, 10 mM Tris-HCl, pH 8.0, 3 mM CaCl₂, 2 mM MgOAc, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, plus protease inhibitors) and 30 µl of high salt buffer (low salt buffer with 800 mM KCl and 1% Nonidet P-40) was added slowly followed by incubation on ice for 30 min. Following centrifugation at full speed for 10 min, the supernatants containing nuclear proteins were used in 10 µl of binding reactions at room temperature containing 5 µg of nuclear extract, 1 µg of poly(dl-dC) (GE Healthcare), 10 mM Tris-HCl, pH 8.0, 150 mM KCl, 0.5 mM EDTA, 0.1% Triton X-100, 12.5% glycerol, 0.2 mM dithiothreitol, and the ³²P-labeled double-stranded oligonucleotide probe corresponding to the region of the TLR2 promoter containing the putative NF-κB-binding site. For competition experiments, unlabeled competitor oligonucleotide was added 10 min prior to addition of labeled probe. For supershift experiments, 1 µg of α-p65 antibody or rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the binding reaction. These reactions were then resolved on a nondenaturing 5% polyacrylamide gel, and visualized with ethidium bromide. PCR products were subcloned using the Topo-TA cloning kit (Invitrogen), and at least 12 clones for each condition were sequenced.

Chromatin Immunoprecipitation (ChIP)—ChIP experiments were performed as described elsewhere (28) with slight modifications. 2 × 10⁷ monocytes were fixed, and chromatin was sonicated to yield fragments between 200 and 1000 bp using the Sonics Vibracell VC130 sonicator at a setting of 25% power. Five 20-s pulses were performed with 30-s rests on ice between each pulse. Polyclonal α-p65, CBP, and p300 antibodies used for ChIP were all purchased from Santa Cruz Biotechnology. The anti-diacytel H3 antisera (specific for histone H3 acetylated at Lys-9 and/or Lys-14) was a generous gift from Dr. Craig Mizzen (University of Illinois). Real time PCR primers used to amplify the endogenous TLR2 promoter were as follows: forward 5’-TCCAGAGTTCCCTCCGCGC-3’ and reverse 5’-GCCCCGGGACTAGGAAGTAAAGC-3’. Primers used to amplify the TLR2 promoter from the luciferase reporter plasmids were as follows: forward (TLR2) 5’-TCAGGTTACTGCTCGGAGTTCC-3’ and reverse (luciferase) 5’-CGTCTTCCATGGTGCTTTACC-3’.

In all experiments 10% of the input chromatin was removed prior to addition of the antibodies and used to normalize the amount of immunoprecipitated DNA.

Flow Cytometry—Monocytes were stained using (R)-phycocerythrin-labeled α-TLR2 antibody (clone T2.5) or an isotype-matched control antibody from eBioscience (San Diego). Cells were run on a Coulter XL flow cytometer and analyzed using Summit analysis software. Mean fluorescence intensity values were used to quantify surface TLR2 levels after subtracting the isotype control background values.

RESULTS

Microbial Agonists Induce TLR2 by a Mechanism Requiring New Transcription and NF-κB—To assay the effects of microbial stimulation on TLR2 gene expression, mRNA and surface protein levels were measured in primary human monocytes stimulated for varying amounts of time with MALP-2 (a lipopeptide TLR2/6 agonist). A previous study has shown that TLR2 mRNA levels in primary monocytes increased independent of any added stimulus during the first few hours of culture following isolation from peripheral blood (22), and this is consistent with our observations (data not shown). To avoid this complication, monocytes were allowed to rest for 24 h following isolation prior to stimulation in all experiments (unless otherwise indicated). Fig. 1 shows that stimulation with MALP-2 caused rapid up-regulation of TLR2 mRNA, which peaked at 2 h, and surface protein levels followed with slightly delayed kinetics peaking at 4 h. This increase was transient, however, as both mRNA and surface protein returned to basal levels within 8 h after addition of the agonist.

To begin characterizing the mechanism of TLR2 up-regulation, monocytes were treated with various TLR2 and TLR4 agonists for 2 h in the presence or absence of a transcriptional inhibitor, actinomycin D. Fig. 2A shows a representative autoradiograph obtained from an RNase protection assay of MALP-2-treated cells. Fig. 2B is a quantification of results from triplicate experiments with MALP-2 Pam₃CSK₄ (a TLR2/1 agonist), or LPS (a TLR4 agonist). These results indicate that TLR2 agonists were the most potent inducers of TLR2 gene expression, whereas the TLR4 agonist showed about one-half the capacity to induce TLR2. Furthermore, actinomycin D completely abolished up-regulation of TLR2 in response to all three stimuli indicating that new transcription is required for TLR2 induction.

Because TLR agonists are known to activate the transcription factor NF-κB (29), we next wished to test the hypothesis that NF-κB is required for the observed agonist-induced TLR2 up-regulation. To this end, cells were treated with MALP-2 in the presence or absence of a pharmacological inhibitor, PDTC, which has been shown to prevent nuclear translocation of NF-κB in human monocytes (30). Fig. 3 shows that PDTC treatment completely abolished TLR2 up-regulation in response to
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MALP-2 indicating that NF-κB activity is required for this process.

The TLR2 Promoter Contains a Putative NF-κB-binding Site—
As our results suggest that NF-κB is involved in TLR2 regulation, we next wished to examine the TLR2 promoter to determine whether a putative binding site for this transcription factor was present. Fig. 4A shows a schematic diagram of the TLR2 gene along with an enlargement of the sequence surrounding the beginning of exon I. A near-consensus NF-κB-binding site (underlined) is found in close proximity to the beginning of exon I. However, a previous characterization of the TLR2 transcriptional start site in freshly isolated monocytes showed that the major start site was located within the NF-κB sequence itself (22) causing doubt about whether this binding site could actually be used by NF-κB. We hypothesized that the transcriptional start site would change under various conditions of culture and stimulation, thus allowing NF-κB access to its binding site. To test this idea, we mapped the transcriptional start site of TLR2 under all conditions used in this study. Fig. 4B shows the results of 5′-RACE analysis using cells that were either freshly isolated or cultured overnight followed by MALP-2 treatment as indicated. Interestingly, the transcriptional start profile was significantly different in each case. To precisely map the sites used, the PCR products were cloned and sequenced. Fig. 4C shows that in freshly isolated cells there is a clear preference for transcription initiation at a site 27 bp downstream of the putative NF-κB site (+27), with promiscuity for additional initiation sites farther upstream. However, in cells cultured for 24 h, two dominant start sites were identified (+27 and +43), and in cells cultured for 24 h and then treated with MALP-2, initiation returned predominantly to the +27 with little utilization of sites farther upstream.

**FIGURE 1.** TLR2 mRNA and cell surface levels increase upon stimulation with bacterial lipopeptide. Primary monocytes were isolated, cultured for 24 h, and then stimulated for the indicated times with 20 ng/ml MALP-2. The mRNA of TLR2 and a housekeeping gene L32 were analyzed by RNase protection assay (A). TLR2 surface levels were assessed by flow cytometry (B). The data were quantified by normalizing mRNA values to that of L32 and by directly measuring mean fluorescence intensity values of surface staining (C). In both cases, the value in resting cells was normalized to 1. Error bars represent the standard deviation from experiments performed in triplicate.

**FIGURE 2.** Microbial agonists up-regulate TLR2, and this requires new transcription. Monocytes were stimulated with 20 ng/ml of the indicated agonists for 2 h, and mRNA levels were measured by RNase protection assay. Some cells were treated as indicated with 10 μg/ml actinomycin D (ActD) for 15 min prior to agonist addition. A, representative autoradiograph of RNase protection assay results. B, quantification of results using PhosphorImaging analysis. TLR2 mRNA levels were normalized to the housekeeping gene L32. Error bars represent the standard deviation from experiments performed in triplicate.

**FIGURE 3.** NF-κB activity is required for TLR2 up-regulation. Monocytes were treated with 20 ng/ml MALP-2 as indicated for 2 h, PDTC, an inhibitor of NF-κB activity, was added as indicated for 1 h prior to MALP-2 addition. Cells were then lysed, and TLR2 mRNA levels were measured by real time RT-PCR. Error bars represent the standard deviation from experiments performed in triplicate.
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We next wished to determine whether mutation of the NF-κB-binding site would affect the position of the TLR2 transcriptional start site. To accomplish this, monocytes were transfected with plasmids containing either the wild-type (WT) TLR2 promoter or one containing a mutated NF-κB-binding site (Mut). The NF-κB-binding site is underlined in Fig. 4A. 24 h later, cells were treated with 20 ng/ml MALP-2 for 2 h as indicated, lysed, and subjected to 5’-RACE analysis. Primers used in the 5’-RACE assay were designed to amplify TLR2-luciferase transcripts from the plasmids but not endogenous TLR2 transcripts. Expected transcription start sites based upon the size of each PCR product are indicated by arrows on the right of the photograph. MW, molecular weight markers.

FIGURE 4. Analysis of the TLR2 transcriptional start site and promoter in primary monocytes. 5’-RACE was used to analyze the TLR2 start site under various conditions, including freshly isolated monocytes (fresh), monocytes cultured for 24 h following isolation (cultured), and monocytes cultured for 24 h following 2 h of MALP-2 treatment (MALP-2). A schematic showing the genomic organization of the TLR2 gene and an enlargement of the sequence surrounding the beginning of exon I. Arrows indicate the six alternative start sites identified. +9 through +43 (numbers designate the distance downstream of the beginning of exon I). B, 5’-RACE PCR products. C, PCR products from C were cloned, and at least 12 clones for each condition were sequenced to identify the precise transcription start site. The number of clones obtained for each condition is shown.

FIGURE 5. Mutation of the NF-κB-binding site affects the transcriptional start profile of TLR2. Primary monocytes were transfected with luciferase reporter plasmids containing either the wild-type (WT) TLR2 promoter or one containing a mutated NF-κB-binding site (Mut). Fig. 5A shows that p65 was recruited to the wild-type TLR2 promoter and chromatin immunoprecipitation assay (ChIP) analysis revealed that NF-κB could bind specifically to this site in activated monocytes, and the identified binding site became more precisely defined with promiscuity for additional sites. These results suggest that the binding of NF-κB to this proximal site in the TLR2 promoter plays a role in determining the position of transcriptional initiation.

NF-κB Binds to Its TLR2 Site in Vivo—Having identified a potential binding site for NF-κB in the TLR2 promoter, we next investigated whether NF-κB could bind specifically to this site using EMSA and ChIP analyses. Nuclear extracts from MALP-2-treated monocytes were used in binding reactions with a probe containing the putative TLR2 NF-κB site (Fig. 6). The results of this experiment show that NF-κB bound to the TLR2 probe in a MALP-2-dependent fashion, and this binding was eliminated by addition of a wild-type (but not an NF-κB mutant) competitor oligonucleotide. Furthermore, addition of an antibody specific for the p65 subunit of NF-κB (but not a nonspecific antibody) induced a supershift. These results indicate that NF-κB is able to specifically interact with the predicted binding site in the TLR2 promoter.

NF-κB Is Recruited to Its Binding Site in the Proximal TLR2 Promoter in Vivo—We next wished to measure the in vivo affinity of NF-κB for its site in the TLR2 promoter. To this end, 293T cells were transfected with either the wild-type or NF-κB mutant TLR2 reporter plasmids used above and subjected to ChIP analysis to monitor NF-κB recruitment to the plasmid. The PCR primer set used for this experiment was designed to amplify only TLR2 DNA from the reporter plasmid and not genomic DNA. Fig. 6C shows that p65 was recruited to the wild-type TLR2 reporter plasmid within 2 h after stimulation with MALP-2, but this recruitment was reduced ~3-fold in cells transfected with the plasmid containing the mutated NF-κB site. These data indicate that NF-κB is recruited to the TLR2 promoter in activated monocytes, and the identified binding site is required for this recruitment.

A TLR2 Reporter Gene Is Not Induced by Stimulation with MALP-2—We next wished to test the ability of TLR2 agonists to induce a reporter construct containing the TLR2 promoter fused to the luciferase gene. To this end, three different cell lines (THP-1, 293T, and SW620) were transiently transfected with either the TLR2 construct or a positive control NF-κB-driven luciferase plasmid and then stimulated with MALP-2 as indicated (Fig. 7). In all three cell lines tested, the TLR2 construct was completely unresponsive to stimulation with MALP-2, whereas the NF-κB-driven reporter was induced.

Overexpression of NF-κB, p65, CBP, and P300 Is Sufficient to Induce TLR2 Expression—The lack of inducibility of the TLR2 reporter gene, taken together with our results showing an antiinflammatory effect of MALP-2, led us to hypothesize that TLR2 induction may occur through a mechanism requiring chromatin remodeling or modification, and this mechanism may not function in the context of the reporter plasmid. Therefore, we hypothesized that transcriptional coactivators such as CBP and p300, which are known to interact with NF-κB, are recruited to the TLR2 promoter resulting in...
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To test this idea, the p65 subunit of NF-κB was overexpressed in 293T cells either alone or in combination with CBP or p300 as indicated, and TLR1, TLR2, and TLR6 mRNA levels were measured (Fig. 8). Results of this experiment show that p65 alone induced TLR2 expression, and co-transfection of p300 (and to a lesser extent CBP) further increased the amount of TLR2 mRNA. TLR1 and TLR6 levels remained unchanged, however, by any combination of expression plasmids indicating that transcriptional activation is specific to TLR2.

NF-κB p65, CBP, and p300 Are Recruited to the TLR2 Promoter Followed by Histone Acetylation—To further investigate the idea that co-activators are recruited leading to histone acetylation following MALP-2 stimulation, ChIP was used to analyze the dynamics of the TLR2 promoter in primary monocytes (Fig. 9A). Results show that p65, CBP, and p300 are all recruited within 30 min, and acetylation of histone H3 occurs within 2 h.

To test directly the hypothesis that acetylation of histones in the TLR2 promoter is required for increased TLR2 transcription, we utilized anacardic acid, a cell-permeable pharmacologic agent shown previously to inhibit CBP/p300 histone acetyltransferase activity (31). Fig. 9B shows that MALP-2-dependent induction of TLR2 mRNA in monocytes pre-treated with anacardic acid was reduced ~3-fold, indicating that histone acetyltransferase activity is indeed required for maximal TLR2 transcription to occur. These results are consistent with the model of NF-κB-mediated recruitment of CBP/p300 leading to histone acetylation and activation of the TLR2 promoter.
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In the decade since the characterization of the first vertebrate TLR (32), intensive study of this receptor family has led to the appreciation of its central role in the innate immune response. We now know that TLRs are capable of triggering a powerful inflammatory reaction in response to numerous microorganisms. The requirement for TLRs in protection from infectious disease has been highlighted by many genetic studies in mice and humans (3). However, despite its clear protective role against numerous infectious agents, uncontrolled TLR signaling can lead to chronic inflammation and, in extreme cases, septic shock. Therefore, it seems logical that strict regulatory mechanisms would exist that allow for appropriate responses to pathogens, but are also able to prevent the TLR-mediated response from leading to uncontrolled inflammation. A simple way in which the immune system could accomplish this is by tightly regulating expression of the TLRs themselves. In fact, in vivo expression of TLR family members, particularly TLR2 and TLR4, has been shown to be modulated in patients with various inflammatory diseases, including rheumatoid arthritis, chronic obstructive pulmonary disease, and atopic dermatitis (14, 15, 33). In addition, many studies have demonstrated an increase in TLR2 expression on monocytes derived from sepsis patients relative to healthy controls (8, 12, 13), and a recent clinical study has shown that increased TLR2 expression levels on blood monocytes from infected patients appears to be predictive of a recurrence of infection (9).

In this study, we have characterized the effects of microbial stimulation on the expression of TLR2 in primary human monocytes. We have found that the TLR2 gene is rapidly induced in response to agonists for both TLR2 and TLR4 indicating that the mechanism involves a signaling pathway shared by both of these receptors. TLR2 mRNA and cell surface receptor levels were both increased suggesting that this change in expression may be functionally significant. It is tempting to conclude from these results that TLR2 is up-regulated to enhance inflammatory responses to infection. However, it has been shown that treatment of monocytes and macrophages with bacterial lipoproteins induces a state of tolerance in which cells become refractory to a second challenge with lipoproteins (34). Therefore, it is possible that induction of the TLR2 gene does not enhance responsiveness to TLR2 agonists. In fact, given the existence of soluble forms of TLR2 that arise from post-translational mechanisms and are capable of modulating TLR2 signaling (35), one could speculate that TLR2 up-regulation would actually dampen sensitivity. Further investigation will be required to address this possibility.

Our results showing induction of the TLR2 gene are consistent with several studies indicating that murine Tlr2 is induced by pro-inflammatory mediators (17, 19). However, data from previous studies on the response of the human TLR2 gene have been somewhat inconsistent, with some groups claiming that the gene is inducible by microbial stimuli (5, 21) and others reporting no effect (7, 22). We believe that the inconsistencies are a result of procedural differences. Haehnel et al. (22) have shown that when primary monocytes are cultured following isolation from peripheral blood, TLR2 mRNA is induced within 2 h independent of any additional stimulus. This is consistent with our observations as well (data not shown). Because the kinetics of this stimulus-independent up-regulation are very similar to those we have observed in this study in response to TLR agonists, it is likely that the effects of microbial stimuli in previous studies have been masked. We have avoided this com-

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**DISCUSSION**

In the decade since the characterization of the first vertebrate TLR (32), intensive study of this receptor family has led to the appreciation of its central role in the innate immune response. We now know that TLRs are capable of triggering a powerful inflammatory reaction in response to numerous microorganisms. The requirement for TLRs in protection from infectious disease has been highlighted by many genetic studies in mice and humans (3). However, despite its clear protective role against numerous infectious agents, uncontrolled TLR signaling can lead to chronic inflammation and, in extreme cases, septic shock. Therefore, it seems logical that strict regulatory mechanisms would exist that allow for appropriate responses to pathogens, but are also able to prevent the TLR-mediated response from leading to uncontrolled inflammation. A simple way in which the immune system could accomplish this is by tightly regulating expression of the TLRs themselves. In fact, *in vivo* expression of TLR family members, particularly TLR2 and TLR4, has been shown to be modulated in patients with various inflammatory diseases, including rheumatoid arthritis, chronic obstructive pulmonary disease, and atopic dermatitis (14, 15, 33). In addition, many studies have demonstrated an increase in TLR2 expression on monocytes derived from sepsis patients relative to healthy controls (8, 12, 13), and a recent clinical study has shown that increased TLR2 expression levels on blood monocytes from infected patients appears to be predictive of a recurrence of infection (9).

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**FIGURE 9.** MALP-2 treatment induces NF-κB and CBP/p300 recruitment to the TLR2 promoter in vivo followed by histone acetylation. A primary monocytes were stimulated with 20 ng/ml MALP-2 for the indicated times and then subjected to ChIP analysis using antibodies to p65, CBP, p300, or acetylated histone H3 as indicated. Immunoprecipitated DNA levels were quantified by real time PCR and normalized to that of resting cells whose value was taken as 1. Error bars represent the standard deviation from experiments performed in triplicate. B, monocytes were treated with 20 ng/ml MALP-2 as indicated for 2 h. One set of cells was treated with 10 μM anacardic acid for 1 h prior to MALP-2 addition. Cells were then lysed, and TLR2 mRNA levels were measured by real time RT-PCR. Values are expressed as fold change relative to unstimulated cells. Error bars represent the standard deviation from experiments performed in triplicate. AA, anacardic acid.
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plication by allowing monocytes to rest in culture for 24 h before addition of agonist, thus allowing us to separate agonist-dependent and -independent effects on TLR2 expression. In our experiments, all cells were cultured for the same amount of time following isolation; therefore, any effects on TLR2 expression could only be a function of added microbial agonist and not the length of time in culture.

In addition to establishing that human TLR2 is up-regulated in response to TLR agonists, we have characterized the mechanism by which this induction occurs. Using pharmacological inhibitors of RNA polymerase and NF-κB, we have shown that TLR2 is transcriptionally activated via a mechanism involving NF-κB. We also determined that a consensus NF-κB-binding site in the proximal promoter of human TLR2 binds NF-κB in vitro and in vivo. Because a previous study indicated that transcriptional initiation of TLR2 occurs at this NF-κB site in freshly isolated monocytes (22), we characterized the effects of culture and stimulation on the position of initiation. We found that in monocytes cultured for 24 h, transcription was initiated either 27 or 43 bp downstream of the NF-κB site, and when stimulated, the only major start site was +27. In addition, we found that mutation of the NF-κB site affected the transcriptional start profile, suggesting that NF-κB can in fact bind to its site in the TLR2 promoter in vivo and affect transcriptional initiation at this gene.

In concordance with the findings of others, no induction of a reporter plasmid driven by the TLR2 promoter was observed following agonist addition in our study (22). The fact that stimulation of TLR2 expression is observed from the genomic TLR2 promoter, but not from a reporter plasmid, is consistent with a role for chromatin remodeling. In this regard, studies have shown that some, but not all, plasmids are capable of being properly chromatinized when transfected into mammalian cells (36). Additionally, a study of the murine Tlr2 promoter has indicated that chromatin remodeling occurs when cells are infected with Mycobacterium avium (37). Therefore, we focused on testing the hypothesis that induction occurs via effects on chromatin structure at the TLR2 promoter.

In recent years, the list of genes shown to be regulated by epigenetic mechanisms such as histone modification, DNA methylation, and nucleosome remodeling has grown steadily, and this list includes numerous genes involved in the immune response (38–40). The transcriptional co-activators CBP and p300 have been shown to activate expression of many genes through their histone acetyltransferase activities (41, 42), and it has been shown that NF-κB, when phosphorylated at serine 276 of the p65 subunit, is capable of recruiting these co-activators to the promoters of genes such as interleukin (IL)-6 to mediate activation in response to pro-inflammatory stimuli (43, 44). The results of our studies indicate that although p65 alone is sufficient to induce the TLR2 gene, co-expression of either CBP or p300 further up-regulates TLR2 transcription. Chromatin immunoprecipitation analysis confirmed that NF-κB, CBP, and p300 are all recruited to the TLR2 promoter in primary monocytes within 30 min following stimulation with MALP-2. After 2 h of stimulation, CBP appeared to remain bound, and the levels of TLR2 promoter-associated NF-κB and p300 were reduced. This, coupled with the observation that the effect of p300 overexpression was much greater than that of CBP, suggests that these two highly homologous proteins may have different functions with respect to TLR2 gene regulation. Recruitment of these proteins is followed by acetylation of histone H3. These results are consistent with a model in which NF-κB translocates to the nucleus upon cell stimulation and binds to its site in the TLR2 promoter. This event is associated with an altered site of transcription initiation and is followed by recruitment of CBP and p300, which act to acetylate histones in the region thus relaxing nucleosomal structure and allowing maximal transcription to occur. The fact that TLR2 induction is sensitive to a cell-permeable pharmacologic inhibitor of CBP/p300 histone acetyltransferase activity provides further support for this model. As NF-κB is activated in response to numerous inflammatory stimuli in a variety of cell types that express TLR2 suggests that this mechanism may be a general one for the induction of TLR2 expression.

In conclusion, our results suggest that although the murine and human TLR2 promoters share little or no homology, they are functionally similar in their response to proinflammatory stimuli. Despite the fact that exon I of TLR2 has been placed in a completely different genomic context in humans, the response to signals activating NF-κB have been conserved indicating that this phenomenon may play an important role in shaping the innate immune response to certain pathogens. Therefore, therapeutics that alter expression of TLR2 may provide an attractive avenue for research into treatments for a host of inflammatory disorders, including, but not limited to, bacterial sepsis.

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