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## Inhibition of p38 MAPK by Glucocorticoids via Induction of MAPK Phosphatase-1 Enhances Nontypeable *Haemophilus influenzae*-induced Expression of Toll-like Receptor 2\*

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Despite the importance of glucocorticoids in suppressing immune and inflammatory responses, their role in enhancing host immune and defense response against invading bacteria is poorly understood. We have demonstrated recently that glucocorticoids synergistically enhance nontypeable *Haemophilus influenzae* (NTHi)-induced expression of Toll-like receptor 2 (TLR2), an important TLR family member that has been shown to play a critical role in host immune and defense response. However, the molecular mechanisms underlying the glucocorticoid-mediated enhancement of TLR2 induction still remain unknown. Here we show that glucocorticoids synergistically enhance NTHi-induced TLR2 expression via specific up-regulation of the MAPK phosphatase-1 (MKP-1) that, in turn, leads to dephosphorylation and inactivation of p38 MAPK, the negative regulator for TLR2 expression. Moreover, increased expression of TLR2 in epithelial cells greatly enhances the NTHi-induced expression of several key cytokines, including tumor necrosis factor- $\alpha$  and interleukins 1 $\beta$  and 8, thereby contributing significantly to host immune and defense response. These studies may bring new insights into the novel role of glucocorticoids in orchestrating and optimizing host immune and defense responses during bacterial infections and enhance our understanding of the signaling mechanisms underlying the glucocorticoid-mediated attenuation of MAPKs.

In the host innate immune system, the surface epithelial cells, situated at the interface with the environment, are continually confronted with microbes and thereby act as the first line of host defense against pathogenic bacteria (1, 2). They recognize the microbial pathogens by a set of germ line-encoded receptors that are referred as pattern-recognition receptors. Toll-like receptors (TLRs)<sup>1</sup> function as the major epithelial

pattern-recognition receptors in recognizing pathogen-associated molecular patterns (3). Activation of TLRs, in turn, leads to induction of direct antimicrobial effector pathways that can result in elimination of the invading pathogens (4). Moreover, activation of TLRs also induces the expression of co-stimulatory molecules and the release of cytokines that instruct the acquired immune response (5).

TLRs are a family of closely related type I transmembrane proteins that resemble the mammalian IL-1 receptor (6, 7). To date, 10 members of the human TLR family have been cloned. Among these, TLR2 has been shown to mediate responses to a variety of Gram-positive products, including peptidoglycan, lipoprotein, lipoteichoic acid, and lipoarabinomannan. In addition, our recent study demonstrated that TLR2 also plays an important role in activation of inflammatory and immune responses by lipoprotein from the Gram-negative bacterium *Haemophilus influenzae* (NTHi) (8), a major cause of otitis media, exacerbation of chronic obstructive pulmonary diseases, and bacterial meningitis (11–13). The importance of TLR2 in host defense was further demonstrated by the studies from knock-out mice showing decreased survival of TLR2-deficient mice after infection with Gram-positive *Staphylococcus aureus* (9). Thus, it is clear that TLR2 plays a crucial role in host defense against both Gram-positive and -negative bacteria. In contrast to its relatively higher expression in lymphoid tissues, TLR2 is, however, expressed at low levels in epithelial cells. Interestingly, our recent studies revealed that TLR2, although expressed at very low levels in unstimulated human epithelial cells, is greatly up-regulated by NTHi via a positive IKK $\beta$ -I $\kappa$ B $\alpha$ -dependent NF- $\kappa$ B pathway and a negative MKK3/6-p38 $\alpha$ / $\beta$  pathway (10).

Glucocorticoids are highly effective in the control of many inflammatory and immune diseases. Their effects are exerted by binding to the intracellular glucocorticoid receptor (GR), which belongs to the family of steroid hormone receptors. Despite the importance of glucocorticoids in suppressing immune and inflammatory responses, their role in enhancing host immune and defense response against invading bacterial pathogens is, however, poorly understood. Moreover, in addition to their wide use in the treatment of immune and inflammatory

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<sup>1</sup> The abbreviations used are: TLR, Toll-like receptor; DEX, dexamethasone; GR, glucocorticoids receptor; NTHi, nontypeable *H. influenzae*; MAPK, mitogen-activated protein kinase; MKP-1, MAPK phosphatase-1; MKK, MAPK kinase; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; RT, reverse transcription; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase.

*enzae*; MAPK, mitogen-activated protein kinase; MKP-1, MAPK phosphatase-1; MKK, MAPK kinase; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; RT, reverse transcription; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase.

diseases, adjunctive glucocorticoid therapy has also been used in a variety of bacterial infections, including bacterial meningitis caused by *H. influenzae*, otitis media, and chronic obstructive pulmonary diseases (11–13). The molecular basis for the beneficial effect of glucocorticoids in bacterial infections still remains unclear. Because of the importance of glucocorticoids in the treatment of immune and inflammatory diseases as well as bacterial infections, we recently investigated the effect of glucocorticoids on NTHi-induced TLR2 expression. Surprisingly, in contrast to their potent anti-inflammatory effect, glucocorticoids synergistically enhance NTHi-induced TLR2 expression. Thus, these data suggest that glucocorticoids may not only suppress but also enhance host immune and defense response and may explain the beneficial effect of glucocorticoids in certain bacterial infections. However, the signaling mechanisms underlying glucocorticoid-mediated synergistic enhancement of TLR2 are still unknown as is the physiological relevance of TLR2 up-regulation.

Here, we show that glucocorticoids synergistically enhance the NTHi-induced TLR2 expression via specific up-regulation of the MAPK phosphatase-1 (MKP-1) (14), which, in turn, leads to dephosphorylation and inactivation of p38 MAPK, the negative regulator for TLR2 expression. Moreover, increased expression of TLR2 in epithelial cells also greatly enhance the NTHi-induced expression of several key cytokines, including TNF- $\alpha$ , IL-1 $\beta$ , and IL-8, thus demonstrating the physiological relevance of TLR2 up-regulation in bacterial pathogenesis. These studies, although rather unexpected, may provide novel insights into the role of glucocorticoids in orchestrating and optimizing host defense and innate immune responses and lead to novel therapeutic strategies for modulating innate immune and inflammatory responses for bacterial infections.

#### EXPERIMENTAL PROCEDURES

**Reagents**—Ro-31-8220 and orthovanadate were purchased from Calbiochem (La Jolla, CA). Dexamethasone and RU486 were purchased from Sigma (St. Louis, MO).

**Bacterial Strains and Culture Conditions**—Nontypeable *H. influenzae* (NTHi) strain 12, a clinical isolate, was used in this study. For making NTHi crude extract, NTHi were harvested from a plate of chocolate agar after overnight incubation and incubated in 30 ml of brain heart infusion broth supplemented with NAD (3.5  $\mu$ g/ml). After overnight incubation, NTHi were centrifuged at 10,000  $\times$  *g* for 10 min, and the supernatant was discarded. The resulting pellet of NTHi was suspended in 10 ml of phosphate-buffered saline and sonicated. Subsequently, the lysate was collected and stored at  $-70^{\circ}\text{C}$ . NTHi whole cell lysate was used in all the experiments, unless otherwise indicated. We chose to use NTHi lysates because of the following reasons: First, NTHi has been shown to be highly fragile and has the tendency to autolyze. Its autolysis can be triggered *in vivo* under various conditions, including antibiotic treatment (16). Therefore, using lysates of NTHi represents a common clinical condition *in vivo*, especially after antibiotic treatment.

**Cell Culture**—Human cervix epithelial cell line HeLa was maintained as described previously (8, 10, 16). Primary human airway epithelial cells (NHBE) (Clonetics, Walkersville, MD) were maintained in Bronchial Epithelial Basal Medium (Clonetics) following the manufacturer's instruction.

**RT-PCR Analysis of MKPs**—Total RNA was isolated from human epithelial cells using a Qiagen kit (Valencia, CA) following the manufacturer's instruction. For the RT reaction, the Moloney murine leukemia virus pre-amplification system (Invitrogen, Rockville, MD) was used. PCR amplification was performed with *Taq* gold polymerase (PerkinElmer Life Sciences, Foster City, CA). The oligonucleotide primers were: MKP-1, 5'-GCT GTG CAG CAA ACA GTC GA-3' and 5'-CGA TTA GTC CTC ATA AGG TA-3'; MKP-3, 5'-AGC GAC TGG AAC GAG AAT ACG-3' and 5'-CAT AAG GTA AGC CAC AGT CAC A-3'; MKP-5, 5'-CCA AAT GCA GCA AGA GTC ACC-3' and 5'-TCA TTG CCA AGG AAC AGG AAG G-3'; MKP-7, 5'-ATC TTT ATC TTG GCT GCC AGC GA-3' and 5'-AGC AGG GAC AGG TTC ATT TGG C-3'; cyclophilin, 5'-CCG TGT TCT TCG ACA TTG CC-3' and 5'-ACA CCA CAT GCT TGC CAT CC-3'.

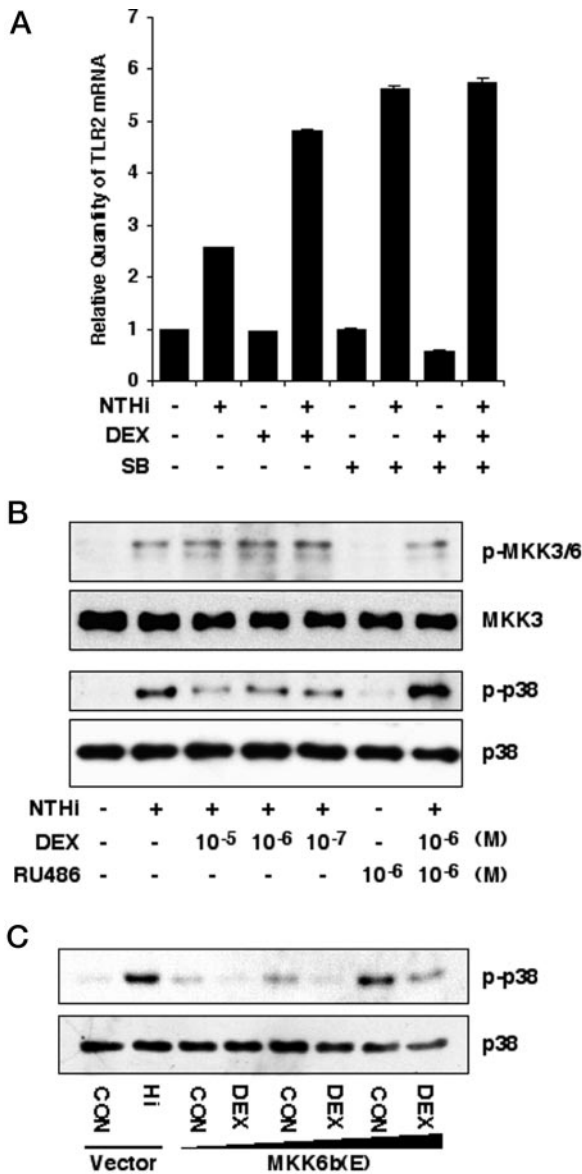
**Real-time Quantitative PCR Analysis of TLR2, TNF- $\alpha$ , IL-1 $\beta$ , and IL-8**—Total RNA was isolated from human epithelial cells as described above. For the RT reaction, TaqMan Reverse Transcription reagents (Applied Biosystems, Foster City, CA) were used. Briefly, the RT reaction (final volume of total 50  $\mu$ l) was conducted for 60 min at  $37^{\circ}\text{C}$  followed by 60 min at  $42^{\circ}\text{C}$  using oligo-dT and random hexamers. PCR amplification was performed with TaqMan Universal Master Mix (Applied Biosystems). In brief, reactions were performed in duplicate containing 2 $\times$  Universal PCR Master Mix, 2  $\mu$ l of template cDNA, 200 nM TLR2 primers (5'-GGCCAGCAAATTACCTGTGTG-3' and 5'-AGGCG-GACATCCTGAACCT-3') and 100 nM TLR2 probe (5'-TCCATCCCAT-GTGCCTGGCC-3'), in a final volume of 25  $\mu$ l, and were analyzed in a 96-well optical reaction plate (Applied Biosystems). TLR2 primers and probes were synthesized by the Applied Biosystems Custom Oligo Synthesis Service. Probes include a fluorescent reporter dye, 6-carboxyfluorescein (FAM), on the 5'-end and labeled with a fluorescent quencher dye, 6-carboxytetramethyl-rhodamine (TAMRA), on the 3'-end to allow direct detection of the PCR product. Reactions were amplified and quantified using an ABI 7700 sequence detector and manufacturer's software (Applied Biosystems). Relative quantity of TLR2 mRNA was obtained using the Comparative CT Method and was normalized using Pre-Developed TaqMan Assay Reagent Human Cyclophilin as an endogenous control (Applied Biosystems) (for details, see user Bulletin #2 for the ABI PRISM 7700 Sequence Detection System, available at [www.appliedbiosystems.com/support/tutorials](http://www.appliedbiosystems.com/support/tutorials)). Briefly, the TaqMan software (Applied Biosystems) was used to calculate a  $C_t$  value for each reaction, where the  $C_t$  value is the point in the extension phase of the PCR reaction that the product is distinguishable from the background. The  $C_t$  values were then normalized for TLR2 amplification by subtracting the  $C_t$  value calculated for cyclophilin, an endogenous control for the amount of mRNA from the same sample, to obtain a  $C_t$  using the following equation:  $C_t \text{ TLR2} - C_t \text{ cyclophilin} = C_t$ . The NTHi- or other inducer-dependent -fold induction of TLR2 was calculated relative to the  $C_t$  value obtained in the unstimulated cells. The normalized TLR2 expression was thus expressed as the relative quantity of TLR2 mRNA (-fold induction). The mRNAs of TNF- $\alpha$ , IL-1 $\beta$ , and IL-8 were measured using pre-developed assay reagent kits (Applied Biosystems) and were then normalized similarly to TLR2.

**Plasmids and Transfections**—The expression plasmids hTLR2 and MKK6 were previously described (8, 10, 19, 20). The expression plasmid of the wild-type MKP-1 was kindly provided by Dr. N. Tonks (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) (14). The antisense full-length MKP-1 construct was described previously (15). All transient transfections were carried out in duplicate for RT-PCR analysis using TransIT-LT1 reagent (Panvera, Madison, WI) following the manufacturer's instruction, unless otherwise indicated. In all transfections with expression plasmids of signaling molecules, an empty vector was used as a control.

**Western Blot Analysis**—To detect MKP-1 up-regulation at the protein level, Western blot analysis was carried out using an antibody against human MKP-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). To assess phosphorylation of p38 and MKK3/6, Western blot analysis was carried out using antibodies against phospho-p38 (Thr-180/182), p38, phospho-MKK3/6, and MKK3/6 (New England BioLabs, Beverly, MA). Phosphorylation of p38 and MKK3/6 were detected as described previously (10, 16).

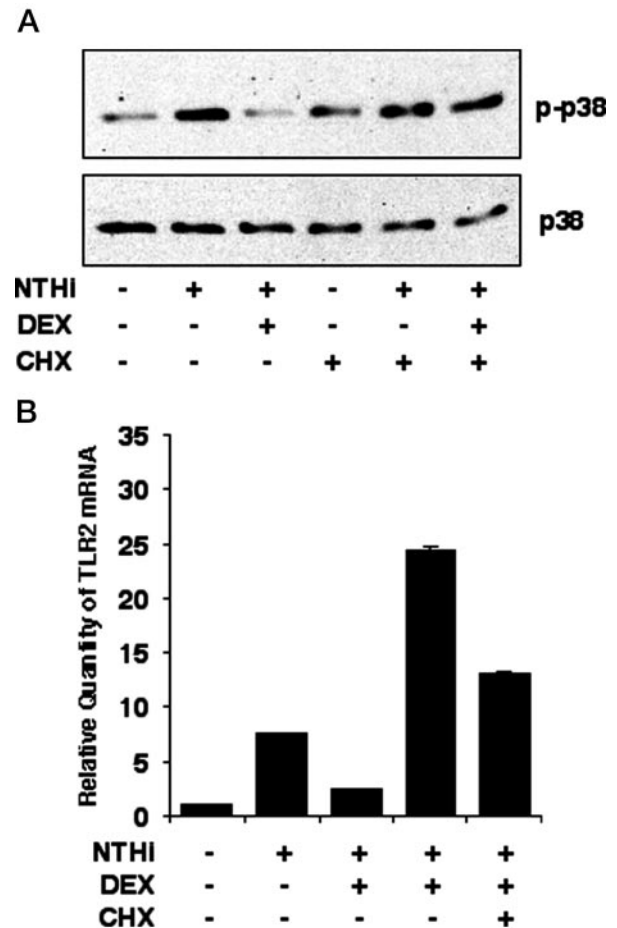
#### RESULTS

**Inhibition of NTHi-induced p38 Phosphorylation by Glucocorticoids Occurs at the Level of p38 Itself**—We have demonstrated recently that glucocorticoids synergistically enhance NTHi-induced TLR2 up-regulation likely via negative cross-talk with the p38 MAPK pathway (10). To determine the molecular mechanisms by which glucocorticoids inhibit NTHi-induced p38 activation that, in turn, leads to enhancement of TLR2 induction, we first reconfirmed the effect of the synthetic glucocorticoid hormone, dexamethasone, on NTHi-induced TLR2 expression in the presence or absence of SB203580, a specific inhibitor for p38 MAPK. As we showed previously, dexamethasone alone synergistically enhanced NTHi-induced TLR2 expression (Fig. 1A). However, dexamethasone no longer greatly enhanced NTHi-induced TLR2 expression if the cells were already pretreated with SB203580, thus confirming that glucocorticoids may enhance NTHi-induced TLR2 expression via inhibition of p38, the negative regulator for TLR2 induc-



**FIG. 1. Inhibition of NTHi-induced p38 phosphorylation by glucocorticoids occurs at the level of p38 itself.** *A*, effects of DEX ( $1 \mu\text{M}$ ) on NTHi-induced TLR2 up-regulation in HeLa cells that were pretreated with SB203580 (SB) ( $5 \mu\text{M}$ ). *B*, dexamethasone (DEX) inhibits NTHi-induced phosphorylation of p38 MAPK, but not MKK3/6. HeLa cells were first pretreated with DEX for 2 h in the presence or absence of RU486 ( $1 \mu\text{M}$ ) and were then stimulated with NTHi for 30 min. NTHi-induced phosphorylation of p38 MAPK and MKK3/6 was then assessed by Western blot analysis using anti-phosphorylated p38 and MKK3/6 antibodies. Equal amounts of proteins were loaded. *C*, dexamethasone inhibits the phosphorylation of p38 MAPK induced by overexpression of a constitutively active form of MKK6 (MKK6b(E)). HeLa cells were first transiently transfected with either an MKK6b(E) expression plasmid or an empty vector and were then treated with  $1 \mu\text{M}$  DEX for 2 h before being lysed for Western blot analysis. The results are representative of three independent experiments. Similar results were also observed in primary human airway epithelial (NHBE) cells. NTHi, nontypeable *H. influenzae*; MKK, MAPK kinase.

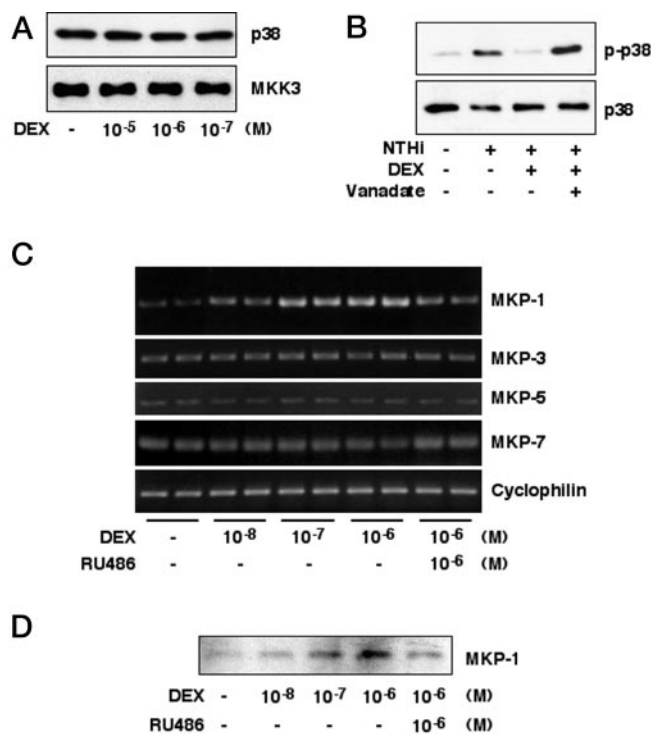
tion. We next sought to investigate whether dexamethasone inhibits NTHi-induced phosphorylation of p38 MAPK by directly inhibiting p38 MAPK or its upstream kinases MKK3/6 (16). As shown in Fig. 1*B*, dexamethasone inhibited NTHi-induced phosphorylation of p38 MAPK, but not MKK3/6, indicating that inhibition of NTHi-induced p38 phosphorylation by glucocorticoids occurs at the level of p38 itself. The inhibitory effect of dexamethasone was blocked by the GR antagonist RU486 at an equimolar concentration, suggesting the inhibi-



**FIG. 2. Glucocorticoids-mediated inhibition of p38 phosphorylation and enhancement of TLR2 expression involves *de novo* protein synthesis.** *A*, the inhibitory effect of dexamethasone on NTHi-induced p38 phosphorylation is antagonized by cycloheximide, a protein synthesis inhibitor. HeLa cells were first pretreated with  $1 \mu\text{M}$  DEX for 2 h in the presence or absence of  $20 \mu\text{M}$  cycloheximide (CHX) and were then treated with NTHi for 30 min. Western blot analysis was carried out to assess the level of the p38 phosphorylation. The results are representative of three independent experiments. *B*, dexamethasone-mediated enhancement of NTHi-induced TLR2 expression is also inhibited by cycloheximide. HeLa cells were first pretreated with  $1 \mu\text{M}$  DEX for 2 h in the presence or the absence of  $20 \mu\text{M}$  CHX and were then treated with NTHi for 3 h. Real-time quantitative PCR analysis was performed to assess TLR2 expression at mRNA level. TLR2 mRNA levels were normalized to the level of cyclophilin that served as an internal control for the amount of RNA used in each reaction. Values are the mean  $\pm$  S.D.;  $n = 3$ .

tory effect of dexamethasone is mediated by the glucocorticoid receptor (GR) (17, 18). Similar to NTHi-induced p38 phosphorylation, the phosphorylation of p38 induced by overexpression of a constitutively active form of MKK6 (MKK6b(E)), an immediate upstream activator of p38 (19–21), was also inhibited by dexamethasone, implicating that the inhibitory effect of dexamethasone may be also applied to p38 phosphorylation activated by other p38 inducers in addition to bacterium NTHi (Fig. 1*C*). Together, these data demonstrate that inhibition of p38 activation by dexamethasone occurs at the level of p38 MAPK, downstream of MKK3/6.

**Glucocorticoids-mediated Inhibition of p38 Phosphorylation Involves *de Novo* Protein Synthesis**—We next sought to determine whether direct interaction between p38 MAPK and GR is involved in glucocorticoid-mediated inhibition of p38 phosphorylation. Interestingly, GR was not found to be directly associated with p38 MAPK (data not shown) as assessed by performing immunoprecipitation experiment using antibody against

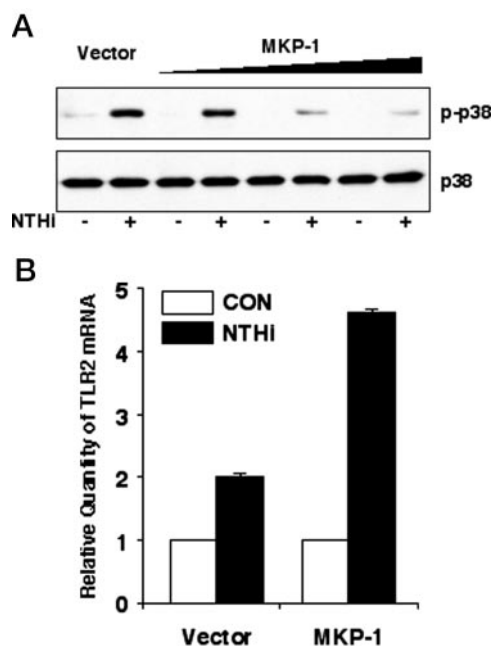


**FIG. 3. Glucocorticoids specifically up-regulate the expression of MKP1.** *A*, the expression of p38 and MKK3 proteins is not affected by dexamethasone. HeLa cells were treated with DEX for 2.5 h before being lysed for Western blot analysis. *B*, glucocorticoid-mediated inhibition of p38 phosphorylation was blocked by orthovanadate, a protein phosphatase inhibitor. HeLa cells were first pretreated with 1  $\mu$ M DEX for 2 h in the presence or the absence of 200  $\mu$ M orthovanadate and were then stimulated with NTHi for 30 min. *C*, dexamethasone strongly up-regulates the expression of MKP-1, but not MKP3, -5, and -7, at the mRNA levels. HeLa cells were treated with DEX for 2.5 h in the presence or the absence of 1  $\mu$ M RU486. RT-PCR analysis was then performed in duplicate to assess MKPs expression at mRNA level. *D*, dexamethasone greatly up-regulates the expression of MKP-1 at the protein level. HeLa cells were treated with DEX for 2.5 h in the presence or absence of 1  $\mu$ M RU486. MKP-1 expression was then assessed by Western blot analysis using an anti-MKP-1 antibody. Equal amounts of proteins were loaded. The results are representative of three independent experiments. Similar results were also observed in primary human airway epithelial (NHBE) cells. *MKP*, MAPK phosphatase.

p38, followed by immunoblot analysis using antibody against GR, or vice versa. Thus, the molecular mechanism underlying GR-mediated inhibition of p38 appears to be independent of a direct physical interaction between GR and p38.

To determine the possible involvement of *de novo* protein synthesis in the action of dexamethasone, we then assessed the effect of dexamethasone on NTHi-induced p38 phosphorylation and TLR2 up-regulation in the presence or absence of the protein synthesis inhibitor cycloheximide. The inhibitory effect of dexamethasone on NTHi-induced p38 phosphorylation was antagonized by cycloheximide, suggesting the requirement for new protein synthesis in the glucocorticoid-mediated inhibition of p38 activation (Fig. 2A). In accordance with these results, dexamethasone-mediated enhancement of NTHi-induced TLR2 expression was also inhibited by cycloheximide (Fig. 2B). Therefore, it is clear that *de novo* protein synthesis is involved in glucocorticoid-mediated inhibition of NTHi-induced p38 phosphorylation and the subsequent enhancement of TLR2 expression.

**Glucocorticoids Specifically Up-regulate the Expression of MKP1**—Based on the evidence for the involvement of new protein synthesis in glucocorticoid-mediated inhibition of p38 activation and enhancement of TLR2 expression, it is logical that glucocorticoids may inhibit p38 activation by either down-

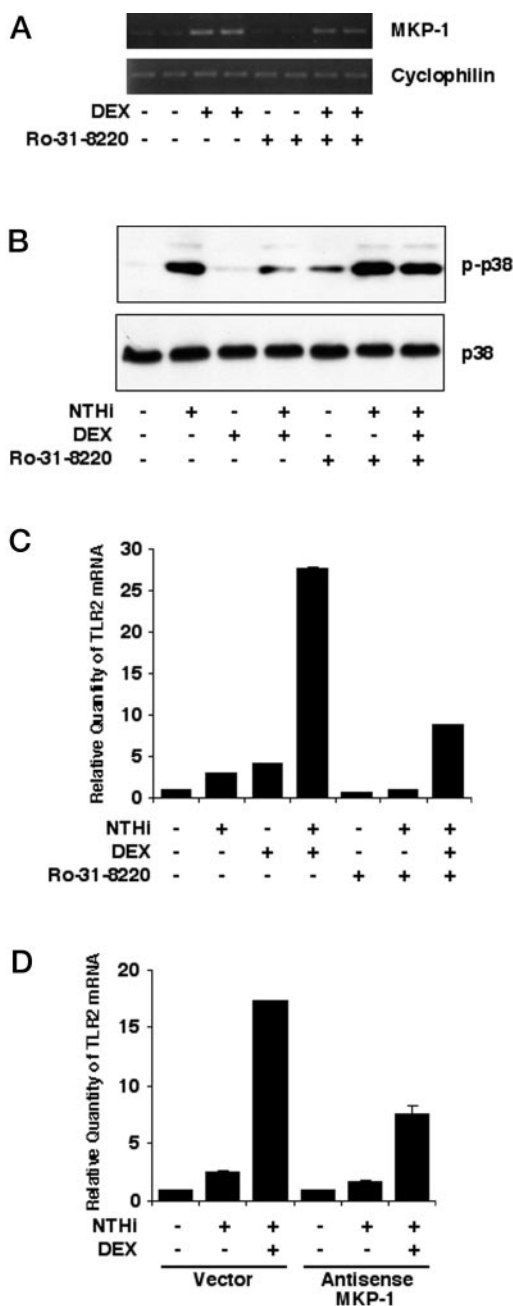


**FIG. 4. Overexpression of MKP1 inhibits NTHi-induced p38 phosphorylation and enhances NTHi-induced TLR2 up-regulation.** *A*, overexpression of MKP-1 abolishes NTHi-induced p38 phosphorylation. HeLa cells were transiently transfected with a wild-type MKP-1 expression plasmid or an empty vector and were then stimulated with NTHi for 30 min before being lysed for Western blot analysis using an anti-phosphorylated p38 antibody. The results are representative of three independent experiments. *B*, NTHi-induced TLR2 expression at the mRNA level is synergistically enhanced by overexpressing MKP-1. HeLa cells were transiently transfected with a wild-type MKP-1 expression plasmid and were then stimulated with NTHi for 3 h. Real-time quantitative PCR was performed to assess TLR2 expression at mRNA level. TLR2 mRNA levels were normalized to the level of cyclophilin that served as an internal control for the amount of RNA used in each reaction. Values are the mean  $\pm$  S.D.;  $n = 3$ . Similar results were also observed in primary human airway epithelial (NHBE) cells.

regulating the expression of p38 protein or up-regulating the inhibitors for p38 phosphorylation and activation. To test the first possibility, we evaluated the effect of dexamethasone on p38 expression at the protein level. As evidenced in Fig. 3A, the expression of p38 protein was not affected by dexamethasone. As expected, no change was observed in MKK3 expression upon dexamethasone treatment. These results have thus led us to investigate whether dexamethasone up-regulates the expression of inhibitors for p38 phosphorylation.

Because protein phosphatases have been shown to play an important role in dephosphorylating p38 (15, 21), we therefore examined whether protein phosphatases are involved in glucocorticoid-mediated inhibition of p38 phosphorylation. We first evaluated the effect of dexamethasone on NTHi-induced p38 phosphorylation in the presence of a protein phosphatase inhibitor, orthovanadate. As shown in Fig. 3B, glucocorticoid-mediated inhibition of p38 phosphorylation was blocked by orthovanadate, thus suggesting the involvement of protein phosphatases.

In review of the known protein phosphatases for p38, a novel class of dual specificity phosphatases, collectively termed MAPK phosphatases (MKPs), represents a major group of protein phosphatases that dephosphorylate and inactivate p38 MAPK (14, 15, 21). To determine whether glucocorticoids up-regulate the expression of MKPs, we next evaluated the effect of dexamethasone on expression of MKPs at the mRNA level by performing RT-PCR analysis. Interestingly, dexamethasone strongly up-regulates MKP-1, but not MKP3, -5, and -7, and the up-regulating effect of dexamethasone was antagonized by



**FIG. 5. Inhibition of MKP-1 attenuates dexamethasone-mediated inhibition of NTHi-induced p38 phosphorylation and enhancement of TLR2 expression.** *A*, Ro-31-8220, an inhibitor for MKP-1 expression, attenuates glucocorticoid-induced up-regulation of MKP-1 at the mRNA level. HeLa cells were treated with DEX for 2.5 h in the presence or the absence of 1  $\mu$ M Ro-31-8220. RT-PCR analysis was then performed in duplicate to assess MKP-1 expression at mRNA level. *B*, the inhibitory effect of dexamethasone on NTHi-induced p38 phosphorylation is antagonized by Ro-31-8220. HeLa cells were first pretreated with 1  $\mu$ M DEX for 2 h in the presence or absence of 1  $\mu$ M Ro-31-8220 and were then stimulated with NTHi for 30 min. The results are representative of three independent experiments. *C*, dexamethasone-mediated enhancement of NTHi-induced TLR2 expression is greatly inhibited by Ro-31-8220. HeLa cells were first pretreated with 1  $\mu$ M DEX for 2 h in the presence or absence of 1  $\mu$ M Ro-31-8220 and were then treated with NTHi for 3 h. Real-time quantitative PCR analysis was performed to assess TLR2 expression at mRNA level. TLR2 mRNA levels were normalized to the level of cyclophilin that served as an internal control for the amount of RNA used in each reaction. Similar results were also observed in primary human airway epithelial (NHBE) cells. *D*, dexamethasone-mediated enhancement of NTHi-induced TLR2 expression is also attenuated by overexpression of an antisense full-length MKP-1 construct. HeLa cells were transiently transfected with either an antisense MKP-1 construct or a control

vector. The transfected cells were then pretreated with DEX for 2 h before being treated with NTHi for 3 h. Real-time quantitative PCR analysis was performed to assess TLR2 expression at mRNA level as described above. Values are the mean  $\pm$  S.D.;  $n = 3$ .

RU486 (Fig. 3C). Consistently, up-regulation of MKP-1 by dexamethasone was also observed at the protein level as assessed by Western blot analysis (Fig. 3D). Thus, it is evident that glucocorticoids indeed up-regulate the expression of MKP-1, a known inhibitor for p38 phosphorylation.

**Overexpression of MKP1 Inhibits NTHi-induced p38 Phosphorylation and Enhances NTHi-induced TLR2 Up-regulation**—To determine whether MKP-1 is involved in glucocorticoid-mediated inhibition of NTHi-induced p38 phosphorylation and the subsequent enhancement of NTHi-induced TLR2 expression, we investigated the effect of overexpressing wild-type MKP-1 on NTHi-induced p38 phosphorylation and TLR2 up-regulation in the absence of dexamethasone. Similar to dexamethasone, overexpression of MKP-1 abolished NTHi-induced p38 phosphorylation (Fig. 4A). Likewise, NTHi-induced TLR2 up-regulation was also greatly enhanced by overexpressing MKP-1 (Fig. 4B), completely mimicking the enhancing effect of dexamethasone. These results strongly suggest an important role for increased MKP-1 expression in glucocorticoid-mediated enhancement of NTHi-induced p38 phosphorylation and TLR2 expression.

**Glucocorticoids Synergistically Enhance NTHi-induced TLR2 Expression via MKP-1-dependent Inhibition of p38 MAPK**—To further confirm the requirement of MKP-1 in glucocorticoid-mediated synergistic enhancement of NTHi-induced TLR2 up-regulation, we first assessed the effect of Ro-31-8220, a chemical inhibitor for MKP-1 expression (22), on dexamethasone-induced MKP-1 up-regulation. As expected, Ro-31-8220 indeed inhibited glucocorticoid-induced up-regulation of MKP-1 expression (Fig. 5A). We next sought to determine whether Ro-31-8220 also blocks the dexamethasone-mediated inhibition of NTHi-induced p38 phosphorylation and enhancement of NTHi-induced TLR2 expression. As shown in Fig. 5B, dexamethasone-mediated inhibition of p38 phosphorylation was abolished by Ro-31-8220. Similarly, dexamethasone-mediated synergistic enhancement of NTHi-induced TLR2 expression was also greatly inhibited by Ro-31-8220 (Fig. 5C). To further confirm whether the up-regulation of MKP-1 expression by glucocorticoids is necessary for the enhancement of NTHi-induced TLR2 expression, we investigated the effect of overexpressing an antisense full-length MKP-1 construct on the glucocorticoid-mediated enhancement of TLR2 induction (15). Interestingly, overexpression of the antisense MKP-1 construct inhibited the dexamethasone-mediated enhancement of NTHi-induced TLR2 expression (Fig. 5D). Collectively, our data demonstrated that glucocorticoids synergistically enhance NTHi-induced TLR2 expression via up-regulation of MKP-1 that, in turn, leads to dephosphorylation and inactivation of p38, a negative regulator for TLR2 expression.

**Overexpression of TLR2 Greatly Enhances NTHi-induced Expression of TNF- $\alpha$ , IL-1 $\beta$ , and IL-8**—Having demonstrated that glucocorticoids synergistically enhance NTHi-induced TLR2 expression via MKP-1-dependent inhibition of p38 MAPK, the physiological relevance of TLR2 up-regulation in epithelial cells is, however, still unclear. Because of the importance of TLR2 in NTHi-induced inflammatory and immune response, we hypothesized that up-regulated TLR2 in human epithelial cells plays an important role in enhancing NTHi-induced expression of key cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-8. To test this hypothesis, we evaluated the effect of overexpression of wild-type TLR2 on NTHi-induced expression of

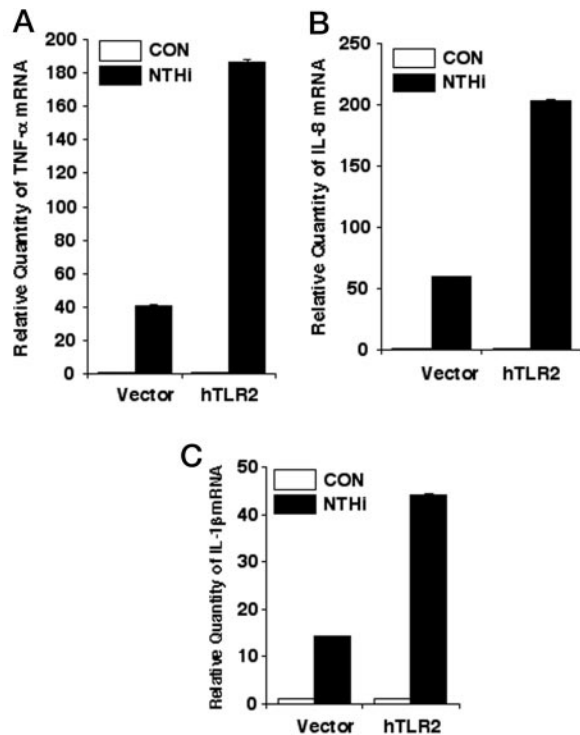


FIG. 6. Overexpression of TLR2 greatly enhances NTHi-induced expression of TNF- $\alpha$ , IL-1 $\beta$ , and IL-8. HeLa cells were transiently transfected with a wild-type hTLR2 expression plasmid or an empty vector and were then stimulated with NTHi for 3 h. Real-time quantitative PCR was performed to assess the expression of TNF- $\alpha$ , IL-1 $\beta$ , and IL-8 at the mRNA levels. Their mRNA levels were normalized to the level of cyclophilin that served as an internal control for the amount of RNA used in each reaction. Values are the mean  $\pm$  S.D.;  $n = 3$ . Similar results were also observed in NHBE cells.

TNF- $\alpha$ , IL-1 $\beta$ , and IL-8 at the mRNA levels by performing real-time quantitative PCR analysis. As shown in Fig. 6, NTHi-induced expression of TNF- $\alpha$ , IL-1 $\beta$ , and IL-8 was greatly enhanced by overexpression of TLR2, thereby demonstrating that up-regulation of TLR2 does play an essential role in NTHi-induced inflammatory and immune response.

#### DISCUSSION

Our studies reveal a previously unknown signaling mechanism by which glucocorticoids synergistically enhance the NTHi-induced expression of TLR2 via specific up-regulation of MKP-1 that, in turn, leads to dephosphorylation and inactivation of p38 MAPK, the negative regulator for TLR2 expression. Moreover, increased expression of TLR2 in epithelial cells greatly enhance the NTHi-induced expression of key cytokines, including TNF- $\alpha$ , IL-1 $\beta$ , and IL-8, thus contributing significantly to host immune response (Fig. 7).

Despite the importance of glucocorticoids in suppressing immune and inflammatory responses, their role in enhancing host immune and defense response against invading bacterial pathogens is poorly understood (23–27). Moreover, the molecular basis for the beneficial role of glucocorticoids in the treatment of bacterial infections such as *H. influenzae*-induced bacterial meningitis also remains unclear. In this study, we reported that glucocorticoids synergistically enhance the expression of TLR2, an important member of the TLR family that has been shown to play a crucial role in host immune and defense response, as evidenced by the studies from knockout mice showing decreased survival of TLR2-deficient mice after infection with Gram-positive *S. aureus* (9). Thus, it appears that glucocorticoids may not only suppress but also enhance host immune and defense response. In addition, our study may

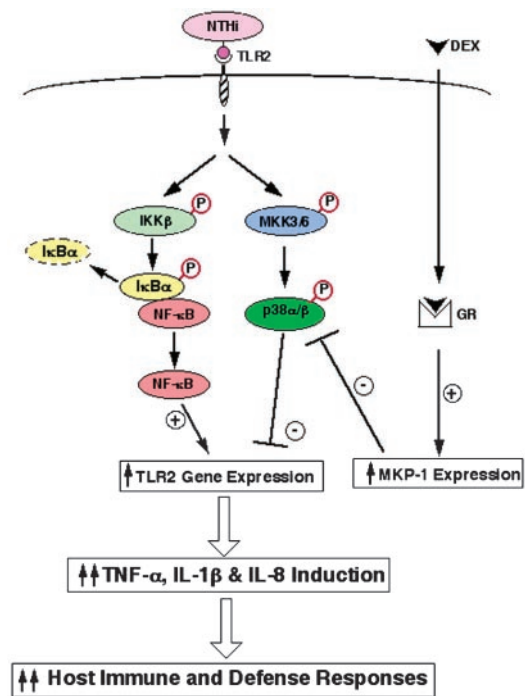


FIG. 7. Schematic representation of the signaling pathways involved in glucocorticoid-mediated enhancement of NTHi-induced TLR2 expression via MKP-1-dependent inhibition of p38 MAPK in human epithelial cells. As indicated, NTHi up-regulates TLR2 expression via a positive IKK $\beta$ -I $\kappa$ B $\alpha$ -dependent NF- $\kappa$ B pathway and a negative MKK3/6-p38 $\alpha$ / $\beta$  pathway. Glucocorticoids, well-known potent anti-inflammatory agents, synergistically enhance NTHi-induced TLR2 expression via specific up-regulation of MKP-1 that, in turn, leads to dephosphorylation and inactivation of p38 MAPK, the negative regulator for TLR2 expression. Moreover, increased expression of TLR2 in epithelial cells greatly enhanced the NTHi-induced expression of several key cytokines, including TNF- $\alpha$ , IL-1 $\beta$ , and IL-8, thereby contributing significantly to host immune and defense responses. DEX, dexamethasone; GR, glucocorticoids receptor; NTHi, nontypeable *H. influenzae*; MAPK, mitogen-activated protein kinase; MKP-1, MAPK phosphatase-1; MKK, MAPK kinase; TLR, Toll-like receptor.

also provide molecular basis for the explanation of the beneficial role of glucocorticoids in certain bacterial infections. What are the molecular mechanisms by which the glucocorticoid-mediated enhancement of TLR2 contributes to host immune and defense response? Given the fact that the TLR2 expression is low in unstimulated epithelial cells, the synergistic enhancement of NTHi-induced TLR2 expression by glucocorticoids will probably contribute to the accelerated immune response by epithelial cells as well as resensitization of epithelial cells to invading pathogens. If so, up-regulation of TLR2 may be one of the positive immune-regulatory mechanisms involved in glucocorticoid-mediated host defense against many bacterial pathogens. Therefore, our study may bring new insights into the novel role of glucocorticoids in orchestrating and optimizing immune functions, including host defense during bacterial infections (23, 24).

Although p38 MAPK has been identified as a target for negative regulation by glucocorticoids (28), the signaling pathway that mediates the down-regulation of p38 by glucocorticoids still remains unclear. Our finding that glucocorticoids inhibit the NTHi-induced phosphorylation and activation of p38 via up-regulation of MKP-1 expression should enhance our understanding of the signaling mechanisms underlying the glucocorticoid-mediated attenuation of MAPKs. In addition to p38, two of the other important MAPK family members, JNK and ERK1/2, have also been identified as targets for negative

regulation by glucocorticoids (29–32). Unlike the involvement of MKP-1 in glucocorticoid-mediated inhibition of p38, glucocorticoid-mediated inhibition of JNK does not require any *de novo* protein synthesis (30). In contrast, the inhibition of ERK1/2 by glucocorticoids does involve new protein synthesis, similar to the inhibition of p38 (29). Thus, the signaling mechanisms underlying the regulation of MAPKs by glucocorticoids are, somehow, diverse.

The molecular mechanisms underlying p38-mediated inhibition of TLR2 induction are still unknown. Because the human TLR2 promoter contains multiple transcription factor binding elements, including Sp1 and Ets-1, and both Sp1 and Ets-1 have been shown to be regulated by p38 signaling pathway (33–35), it is likely that p38-dependent activation of these transcription factors may be involved in p38-mediated inhibition of TLR2 induction. In addition, because TLR2 promoter also contains some uncharacterized regulatory elements (33), these elements may also play a role in transcriptional inhibition of TLR2. Moreover, based on a previous report (36) that p38 negatively regulates ERK1/2 activity and our recent data<sup>2</sup> that demonstrate the positive involvement of ERK1/2 in NTHi-induced TLR2 expression, it is possible that p38 may mediate inhibition of TLR2 induction via negative cross-talk with ERK1/2 pathway. Finally, it is also likely that activation of p38 may be negatively involved in TLR2 induction through down-regulation of additional, as yet unidentified, positive signaling pathways.

In this study, we have shown that glucocorticoids synergistically enhance the NTHi-induced TLR2 expression via specific up-regulation of MKP-1. However, it should be noted that a direct transcriptional activation of TLR2 by glucocorticoids may also partly contribute to the synergistic enhancement of TLR2, because inhibition of MKP-1 by Ro-31-8220 or overexpressing the antisense MKP-1 construct does not completely abolish the synergistic enhancement of TLR2 and glucocorticoids alone also yield severalfold induction of TLR2 expression. Recent success in cloning the regulatory region of human TLR2 will help to elucidate the detailed transcriptional regulatory mechanisms involved in TLR2 regulation (33). In addition, future studies will also focus on identifying other positive signaling pathways involved in TLR2 induction that cannot be inhibited by glucocorticoids. Finally, the molecular mechanisms underlying p38-mediated inhibition of TLR2 induction will also be further investigated. These studies may lead to novel therapeutic intervention for modulating host defense and innate immune and inflammatory responses in bacterial infections.

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<sup>2</sup> A. Imasato and J. D. Li, unpublished results.



**MECHANISMS OF SIGNAL  
TRANSDUCTION:**

**Inhibition of p38 MAPK by  
Glucocorticoids via Induction of MAPK  
Phosphatase-1 Enhances Nontypeable  
*Haemophilus influenzae*-induced  
Expression of Toll-like Receptor 2**

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