Glucocorticoids Synergistically Enhance Nontypeable Haemophilus influenzae-induced Toll-like Receptor 2 Expression via a Negative Cross-talk with p38 MAP Kinase

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Running Title: Synergistic Regulation of TLR2 by Glucocorticoids and NTHi
SUMMARY

The recognition of invading microbes followed by the induction of effective innate immune response is crucial for host survival. Human surface epithelial cells are situated at host-environment boundaries, and thus act as the first line of host defense against invading microbes. They recognize the microbial ligands via Toll-like receptors (TLRs) expressed on the surface of epithelial cells. TLR2 has gained importance as a major receptor for a variety of microbial ligands. In contrast to its high expression in lymphoid tissues, TLR2 is expressed at low level in epithelial cells. Thus, it remains unclear whether the low amount of TLR2 expressed in epithelial cells is sufficient for mediating bacteria-induced host defense and immune response and whether TLR2 expression can be up-regulated by bacteria during infection. Here, we show that TLR2, although expressed at very low level in unstimulated human epithelial cells, is greatly up-regulated by NTHi, an important human bacterial pathogen causing otitis media and chronic obstructive pulmonary diseases. Activation of IKKβ-1κBα-dependent NF-κB pathway is required for TLR2 induction, whereas inhibition of MKK3/6-p38α/β pathway leads to enhancement of NTHi-induced TLR2 up-regulation. Surprisingly, glucocorticoids, well-known potent anti-inflammatory agents, synergistically enhance NTHi-induced TLR2 up-regulation likely via a negative cross-talk with the p38 MAP kinase pathway. These studies may bring new insights into the role of bacteria and glucocorticoids in regulating host defense and immune response and lead to novel therapeutic strategies for modulating innate immune and inflammatory responses for otitis media and chronic obstructive pulmonary diseases.
INTRODUCTION

Effective host defense against invading microorganisms requires the detection of foreign pathogens and the rapid deployment of an antimicrobial effector response (1). In host innate immune system, the surface epithelial cells are situated at host-environment boundaries, and thus act as the first line of host defense against pathogenic bacteria (2). They recognize the invading bacteria by directly interacting with pathogen-associated molecular patterns (PAMPs) on a variety of bacteria via Toll-like receptors (TLRs) expressed on the host (3). Activation of TLRs, in turn, leads to induction of direct antimicrobial activity that can result in elimination of the invading pathogen before a full adaptive immune response takes effect (4). In addition, activation of TLRs is also a prerequisite for the triggering of acquired immunity (5). TLRs are type I transmembrane receptors with leucine-rich repeats in the extracellular domains and cytoplasmic domains that resemble the mammalian IL-1 receptor (IL-1R) (6, 7). To date, 10 members of the human TLR family have been cloned. Of these, TLR2 and TLR4 have been well studied. While TLR4 seems to be mainly involved in Gram-negative bacteria lipopolysaccharide (LPS) signaling, TLR2 can respond to a variety of Gram-positive products, including peptidoglycan, lipoprotein, lipoteichoic acid, and lipoarabinomannan. The importance of TLR2 in host defense was further supported by the studies from knockout mice showing decreased survival of TLR2-deficient mice after infection with Gram-positive S. aureus (8). Moreover, our recent study demonstrated that TLR2 also plays an important role in activation of NF-κB by lipoprotein from the Gram-negative bacterium nontypeable Haemophilus influenzae (NTHi), a major cause of otitis media and exacerbation of chronic obstructive pulmonary diseases (COPD) (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 expressed at low level in epithelial cells. Given the low expression of TLR2 in unstimulated human epithelial cells,
it remains unclear whether the low amount of TLR2 expressed in epithelial cells is sufficient for mediating NTHi-induced inflammatory response and whether TLR2 expression can be up-regulated by bacteria in human epithelial cells during bacterial infections. Here, we explore the possibility that NTHi up-regulates TLR2 in human epithelial cells via activation of specific signaling pathways. Our studies reveal that TLR2, although expressed at very low level in unstimulated human epithelial cells, is greatly up-regulated by NTHi. Activation of IKKβ-IKKα-dependent NF-κB pathway is required for TLR2 induction, whereas inhibition of MKK3/6-p38α/β pathway leads to enhancement of NTHi-induced TLR2 up-regulation. Moreover, glucocorticoids, well-known potent anti-inflammatory agents, synergistically enhance NTHi-induced TLR2 up-regulation likely via a negative cross-talk with the inhibitory p38 MAP kinase pathway. These studies, although rather unexpected, may provide novel insights into the role of bacteria and glucocorticoids in regulating host defense and innate immune responses and lead to novel therapeutic strategies for modulating innate immune and inflammatory responses for otitis media and COPD.
MATERIALS AND METHODS

Reagents. Caffeic acid phenethyl ester (CAPE), MG-132 and SB203580 were purchased from Calbiochem (La Jolla, CA). Dexamethasone and RU486 were purchased from Sigma (St. Louis, MO).

Bacterial Strains and Culture Conditions. Nontypeable Haemophilus 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 (BHI) broth supplemented with NAD (3.5 µg/ml). After overnight incubation, NTHi were centrifuged at 10,000 x 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°C. NTHi whole cell lysate was used in all the experiments, unless otherwise indicated.

Cell Culture. Human cervix epithelial cell line HeLa was maintained as described (9, 14). Human middle ear epithelial cell line HMEEC-1 derived by HPV immortalization of primary human middle ear epithelial cells and primary human airway epithelial cells (NHBE) (Clonetics, Walkersville, MD) were maintained in Bronchial Epithelial Basal Medium (BEBM) (Clonetics) following manufacturer’s instruction.

RT-PCR Analysis of TLR2. Total RNA was isolated from human epithelial cells using a Qiagen kit (Valencia, CA) following manufacturer’s instruction. For the RT reaction, the MMLV preamplification system (Life Technologies, Inc., Rockville, MD) was used. PCR amplification was performed with Taq gold polymerase (Perkin Elmer, Foster City, CA) for 32 cycles at 95 °C for 60 s, 63 °C for 60 s, and 72 °C for 60 s (for TLR2) and 32 cycles at 95 °C for 60 s, 50 °C for 60 s, and 72 °C for 60 s (for cyclophilin). The oligonucleotide
primers were: TLR2, 5’-GCCAAAGTCTTGATGGATTG-3’ and 5’-TTGAAGTTCTCCAGCTCCTG-3’; cyclophilin, 5’-CCGTGTTCGACATTG-3’ and 5’-ACACCACATGCTTGCCATCC-3’.

Real-time Quantitative PCR Analysis of TLR2 and TLR4. Total RNA was isolated from human epithelial cells as described above. For the RT reaction, TaqMan Reverse Transcription Regents (Applied Biosystems, Foster City, CA) were used. Briefly, the RT reaction (final volume of total 50 µl) was conducted for 60 min at 37 °C followed by 60 min at 42 °C using oligo-dT and random hexamers. PCR amplification was performed with TaqMan Universal Master Mix (Applied Biosystems, CA). In brief, reactions were performed in duplicate containing 2X Universal PCR Master Mix, 2 µl of template cDNA, 200 nM of TLR2 primers (5’-GGCCAGCAATTACCTGTGTG-3’ and 5’-AGGCGGACATCCTGAACCT-3’) and 100 nM of TLR2 probe (5’-TCCATCCCATGTGCGTCG-3’) in a final volume of 25 µl, and were analyzed in 96-well optical reaction plate (Applied Biosystems). TLR2 primers and probes were synthesized by Applied Biosystems Custom Oligo Synthesis Service. Probes include a fluorescent reporter dye, FAM, on the 5’ end and labeled with a fluorescent quencher dye, 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 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 under http://www.appliedbiosystems.com/support/tutorials). Briefly, the TaqMan software (Applied Biosystem) was used to calculate a Ct value for each reaction, where the Ct value is the point in the extension phase of the PCR reaction that the product is distinguishable
from the background. The Ct values were then normalised for TLR2 amplification by subtracting the Ct value calculated for cyclophilin, an endogenous control for the amount of mRNA from the same sample, to obtain a Ct using the following equation: Ct TLR2 – Ct cyclophilin = Ct. The NTHI- or other inducer-dependent fold induction of TLR2 was calculated relative to the Ct value obtained in the unstimulated cells. The normalized TLR2 expression was thus expressed as relative quantity of TLR2 mRNA (fold induction).

**TLR4 mRNA was measured using TLR4 primers (5’–CCAGTGAGGATGATGCCAGGAT-3’ and 5’-GCCATGGCTGGGATCAGAGT-3’)** and a TLR4 probe (5’-TGTCTGCCTCGGCCTGCGC-3’) and was then normalised similarly to TLR2.

**Plasmids, Transfections, and Luciferase assays.** The expression plasmids IκBα, IκBα(S32/36A), IKKβ(K49A), fp38α(AF) and wild-type (WT), fp38β2(AF) and WT, MKK3b(A), MKK6b(A), and phGR were previously described (9, 14, 15, 20). The expression plasmid of p65 was kindly provided by A.S. Baldwin (University of North Carolina, Chapel Hill). The reporter construct NF-κB luc was generated as described (9). It contains three copies of the NF-κB site from the IL-2 receptor (α) promoter using the following oligonucleotides: 5’-TCGAGACGGCAGGGGAATCTCCCTCCG-3’ and 3’-CTGCCGTCCCTTACAGGAGAGGCAGCT-5’.

The reporter construct was sequenced to verify number and orientation of inserted oligonucleotides. All transient transfections were carried out in duplicate for RT-PCR analysis and in triplicate for luciferase assays using TransIT-LT1 reagent (Panvera, Medison, WI) following manufacturer’s instruction, unless otherwise indicated. In all co-transfections with expression plasmids of signaling molecules, an empty vector was used as a control.
**Immunofluorescent Staining.** Cells were cultured on 4-chamber microscope slides. After NTHi treatment, the cells were fixed in paraformaldehyde solution (4%), incubated with mouse anti-p65 NF-κB mAb for 1 h (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Primary antibody was detected with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Inc.). Samples were viewed and photographed using a Zeiss Axiophot microscope.

**Western Blot Analysis.** To detect TLR2 up-regulation at protein level, Western blot analysis was carried out using antibodies against human TLR2. To ensure the specificity, two antibodies against human TLR2 from different sources were used, including a polyclonal antibody to TLR2 (H-175) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and a monoclonal antibody to TLR2 (IMG-319) (IMGENEX, San Diego, CA). HeLa cells transfected with human wild-type TLR2 expression plasmid served as a positive control for TLR2 expression. To assess phosphorylation of p38, Western blot analysis was carried out using antibodies against phospho-p38(Thr180/182) and p38 (New England Biolabs, Beverly, MA). Phosphorylation of p38 was detected as described (9, 14).

**Immunohistochemical analysis of human TLR2 expression.** Celloidin embedded sections of normal and diseased mucosa from archival temporal bone specimens were obtained from the House Ear Institute’s Temporal Bone Collection. Following decelloidination with ether and absolute ethanol, non-specific binding sites on the slides were blocked using normal goat serum. The sections were then subjected to antigen retrieval (Zymed Laboratories, South San Francisco, CA) and incubated with polyclonal antibody against human TLR2 (H-175) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Signals were detected by the avidin/biotin complex (ABC) method (Zymed Laboratories).
RESULTS

NTHi up-regulates TLR2, but not TLR4, in human epithelial cells

We first examined whether NTHi up-regulates TLR2 in human epithelial cells. We previously showed that TLR2 was expressed at low level in a variety of human epithelial cells by RT-PCR analysis (9). As shown in Fig. 1A, the expression of TLR2 was very low in HeLa (human cervix epithelial) cells, but was up-regulated by NTHi. Similar results were also observed in HMEEC-1 (human middle ear epithelial) and primary human bronchial epithelial (NHBE) cells (data not shown). We next sought to confirm whether NTHi up-regulates TLR2 by performing real-time quantitative PCR analysis. NTHi indeed strongly up-regulates TLR2 mRNA in HeLa, HMEEC-1 and NHBE cells in a time-dependent manner (Fig. 1B and 1C). Because TLR4 represents another important member of TLR family, we also evaluated the effect of NTHi on TLR4 expression in human epithelial cells. Interestingly, TLR4 did not appear to be strongly up-regulated (Fig. 1D). To determine whether up-regulation of TLR2 mRNA is accompanied by elevated TLR2 protein, Western blot analysis was carried out using antibodies against human TLR2. As shown in Fig. 1E, up-regulation of TLR2 was also observed at protein level. To further confirm whether TLR2 protein is elevated in diseased tissue in vivo, immunohistochemical analysis of TLR2 protein was performed in human middle ear tissues obtained from patients with chronic otitis media and normal individuals, respectively. In comparison to that from normal individuals, the expression of TLR2 protein is indeed higher in epithelial cells of middle ear tissue from patients with chronic otitis media (Fig. 1F). Taken together, these data demonstrate that TLR2, but not TLR4, is up-regulated in human epithelial cells by NTHi.
IKKβ-IκBα-dependent translocation and activation of NF-κB is required for NTHi-induced TLR2 up-regulation

Having demonstrated that TLR2 is up-regulated in human epithelial cells by NTHi, still unknown is which intracellular signaling pathways are involved. Based on the essential involvement of NF-κB in regulating the expression of large numbers of genes involved in immunity and inflammation and our recent study showing the activation of NF-κB by NTHi(9, 10), we determined the role of NF-κB in NTHi-induced TLR2 up-regulation by using caffeic acid phenethyl ester (CAPE), a chemical inhibitor that is known to specifically block the translocation of p65 without affecting IκBα degradation (11). As shown in Fig. 2A, CAPE greatly reduced NTHi-induced TLR2 up-regulation, suggesting that activation of NF-κB is involved in NTHi-induced TLR2 up-regulation. Because disruption of NF-κB:IκBα complex is required for NF-κB nuclear translocation and activation, we next determined the requirement of IκBα degradation by assessing the effect of proteasome inhibitor MG-132 and overexpression of a transdominant mutant of IκBα on NTHi-induced TLR2 up-regulation (12, 13). As expected, MG-132 and overexpression of a transdominant mutant IκBα markedly inhibited TLR2 up-regulation (Fig. 2A). Concomitantly, NTHi-induced NF-κB activation and translocation was also blocked by MG-132 in the same epithelial HeLa cells (Fig. 2B and 2C). On the basis of a recent report that IκB kinase β (IKKβ) acts as an immediate upstream kinase of IκBα, we investigated the role of IKKβ in NTHi-induced TLR2 up-regulation (10). Cotransfection with a dominant-negative mutant form of IKKβ [IKKβ(K44A)] abrogated NTHi-induced TLR2 up-regulation (Fig. 2D). We further confirmed the involvement of NF-κB by transfecting the epithelial cells with an expression plasmid of wild-type p65, the major subunit of NF-κB. Interestingly, expression of wild-type p65 induced TLR2 mRNA in a dose-dependent manner (Fig. 2E). As expected, expression of p65 also induced NF-κB activation (Fig.
Collectively, these findings clearly demonstrated that the IKKβ-IκBα-dependent translocation and activation of NF-κB is required for NTHi-induced TLR2 up-regulation in human epithelial cells.

Activation of MKK3/6-p38α/β MAP kinase pathway is negatively involved in NTHi-induced TLR2 expression

Many cellular stress stimuli can activate both NF-κB and p38 MAP kinase modules. Because of this overlap as well as our recent report that NTHi strongly activates p38 MAP kinase (9, 14, 15), we investigated whether p38 MAP kinase is also involved in NTHi-induced TLR2 up-regulation. Surprisingly, the pyridinyl imidazole SB203580, a highly specific inhibitor for p38 MAP kinase greatly enhanced NTHi-induced TLR2 up-regulation in a dose-dependent manner (16), suggesting that activation of p38 MAP kinase may be negatively involved in NTHi-induced TLR2 up-regulation (Fig. 3A). Moreover, overexpression of a dominant-negative mutant form of either p38α[fp38α(AF)] or p38β[fp38β(AF)] also enhanced the NTHi-induced TLR2 expression, whereas overexpression of a wild-type p38α or p38β reduced it, thereby further supporting the negative involvement of p38 MAP kinase (Fig. 3B). As immediate upstream kinases of p38α and β, two MAP kinase kinases (MKK3 and MKK6) have been identified (15). To further investigate whether activation of MKK3/6 is also negatively involved in NTHi-induced TLR2 up-regulation, a dominant-negative mutant form of either MKK3 [MKK3b(A)] or MKK6 [MKK6b(A)] was transfected into HeLa cells. The NTHi-induced TLR2 up-regulation was enhanced (Fig. 3C). Thus, we concluded from this data that activation of MKK3/6-p38α/β MAP kinase pathway may be negatively involved in NTHi-induced TLR2 up-regulation.
Glucocorticoids synergistically enhance NTHi-induced TLR2 up-regulation via a likely negative cross-talk with p38 MAP kinase pathway

Glucocorticoids have been used widely as anti-inflammatory agents. They exert their anti-inflammatory effects by down-regulating NF-κB-dependent transcription of a variety of genes involved in the inflammatory response (17). Since we have shown that activation of NF-κB is required for NTHi-induced TLR2 up-regulation, it is therefore logical that glucocorticoids may attenuate NTHi-induced TLR2 up-regulation. To test this, we first evaluated the effect of dexamethasone (DEX), a glucocorticoid analog in human epithelial cells, on NTHi-induced up-regulation of TLR2 mRNA. Surprisingly, DEX synergistically enhanced NTHi-induced TLR2 up-regulation at mRNA level in a dose-dependent manner (Fig. 4A). Concomitantly, Western blot analysis showed that DEX also synergistically enhanced NTHi-induced TLR2 up-regulation at protein level (Fig. 4B). These results are rather unexpected, because they were in sharp contrast to the inhibitory effect of DEX on cytokine production (18). Additionally, RU486, a synthetic antiglucocorticoid that acts as a competitor against binding to the glucocorticoid receptor (GR), counteracted the enhancing effect of DEX on NTHi-induced TLR2 up-regulation, suggesting the involvement of GR (Fig. 4C) (19). Moreover, overexpression of wild-type GR in HeLa cells greatly enhanced the synergistic effect of DEX on NTHi-induced TLR2 up-regulation, confirming the involvement of GR (Fig. 4D) (20). Note that a maximal response was observed with 1 µM DEX. Together, these results indicate that DEX synergistically enhanced NTHi-induced TLR2 up-regulation at both mRNA and protein levels via a GR-dependent mechanism.

One key issue that has yet to be addressed is how DEX synergistically enhances NTHi-induced TLR2 up-regulation. Because our results in Fig. 3A and B indicated that inhibition of p38 MAP kinase also synergistically enhanced NTHi-induced TLR2 up-
regulation and glucocorticoids have been shown to inhibit p38 MAP kinase activity (21), we therefore postulated that glucocorticoids may enhance NTHi-induced TLR2 up-regulation via a negative cross-talk with p38 MAP kinase pathway. Our hypothesis was first supported by the result shown in Fig. 4E (upper panel) that DEX no longer enhanced NTHi-induced TLR2 up-regulation if the cells were already pretreated with SB203580, suggesting that DEX and SB203580 may target the same p38 MAP kinase signaling pathway. However, considering that the result was observed with 5 µM SB203580 and 1 µM DEX, a dose shown to elicit a maximal response when used alone, we can not rule out the possibility that the system was already working at its maximal level. We therefore studied the effect of low doses of both SB203580 and DEX on NTHi-induced TLR2 expression. Interestingly, when 0.1 µM DEX was added to the cells that were already pretreated with 0.1 µM SB203580, NTHi-induced TLR2 up-regulation was further enhanced (Fig. 4E, lower panel), thereby suggesting that their ability to enhance TLR2 up-regulation is likely additive to each other. However, it is still unclear whether or not DEX indeed additively enhances TLR2 up-regulation via inhibition of p38 MAP kinase. We therefore investigated the direct effect of DEX on NTHi-induced phosphorylation of p38 MAP kinase. As shown in Fig. 4F, NTHi-induced phosphorylation of p38 was attenuated by DEX pretreatment. Thus, our data demonstrated that glucocorticoids synergistically enhance NTHi-induced TLR2 up-regulation likely via a negative cross-talk with p38 MAP kinase pathway, although our data do not preclude the possibility that a direct protein-DNA interaction between GR and glucocorticoid-response element (GRE) in the regulatory region of the TLR2 gene may also contribute to this synergistic regulation.
DISCUSSION

From what we have shown above, it is evident that TLR2, although expressed at very low level in unstimulated human epithelial cells, is greatly up-regulated by NTHi via a positive IKKβ-IKBa-dependent NF-κB pathway and a likely negative M KK3/6-p38 α/β pathway. The up-regulation of TLR2 by NTHi appears to be consistent with the function of a Drosophila Toll protein whose expression is also up-regulated by a pathogen challenge (22). Our finding may have several important implications in host defense and immune response against bacteria. First, the very low expression of TLR2 we observed in unstimulated epithelial cells is likely to be an important aspect of TLR2 function because under limiting conditions, cellular responses to PAMPs could be more stringently regulated by controlling the amounts of TLR protein produced (23). Second, the increased TLR2 expression will probably contribute to the accelerated immune response by epithelial cells as well as resensitization of epithelial cells to invading pathogens. If so, regulation of TLR2 expression may be one of the immune-regulatory mechanisms commonly involved in host defense against many bacterial strains. Finally, the observation that TLR2 expression is up-regulated by NTHi suggests that invading bacteria can not only initiate the host immune response, but can also modulate the eventual responsiveness of epithelial cells to the invading bacteria by regulating the TLR2 expression level (24). Thus, these observations offer a new insight for fully understanding the important role of TLR2 in epithelial cell defense and immune response against invading bacteria.

Another major interesting finding in this study is the synergistic enhancement of NTHi-induced TLR2 up-regulation by glucocorticoids via a negative cross-talk with the inhibitory p38 MAP kinase signaling pathway. This result, although rather unexpected, may provide a novel insight into the role of glucocorticoids in host defense and inflammatory responses in the pathogenesis of infectious diseases. Recently, it has been
shown that TLR2-deficient mice are highly susceptible to bacterial infection, demonstrating a critical role of TLR2 in host defense against invading bacteria (8). Therefore, the synergistic enhancement of NTHi-induced TLR2 expression by glucocorticoids would undoubtedly contribute to the host defense against bacteria. Since we also provided direct evidence that activation of NF-κB is involved in NTHi-induced TLR2 up-regulation and glucocorticoids are widely known to inhibit NF-κB activity (17, 18), a critical question that has yet to be answered is whether glucocorticoids also inhibit NTHi-induced NF-κB activation, and if so, what mechanisms account for this synergistic enhancement of TLR2 expression. To determine whether glucocorticoids inhibit NF-κB activity, we directly evaluated the effect of glucocorticoids on NTHi-induced NF-κB-dependent promoter activity by using luciferase reporter plasmid in HeLa cells. As expected, glucocorticoids reduced NTHi-induced NF-κB activation by ≈ 70-80% (Fig. 5). Therefore, it may well be that additional positive signaling pathways that can not be inhibited by DEX, together with the direct induction of TLR2 by DEX as well as the inhibition of the negative p38 MAP kinase pathway by DEX, are responsible for the synergistic up-regulation of TLR2 by NTHi and DEX. Thus, the model of the synergistic up-regulation of TLR2 by NTHi and glucocorticoids has become more complex (Fig. 6). Future studies will focus on identifying other positive signaling pathways involved in TLR2 induction. Cloning of the regulatory region of human TLR2 will help to elucidate the transcriptional regulatory mechanisms involved in TLR2 up-regulation. These studies may lead to novel therapeutic intervention for modulating host defense and innate immune and inflammatory responses for otitis media and COPD.
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1 The abbreviations used are: CAPE, Caffeic acid phenethyl ester; COPD, chronic obstructive pulmonary disease; DEX, dexamethasone; GR, glucocorticoids receptor; GRE, glucocorticoid-response element; IKK, IκB kinase; NTHi, Nontypeable Haemophilus influenzae; MAP kinase, mitogen-activated protein kinase; MKK, MAP kinase kinase; TLR, Toll-like receptor;
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FIGURE LEGENDS

Figure 1. NTHi up-regulates TLR2, but not TLR4, in human epithelial cells. (A) The expression of TLR2 at mRNA level was measured by RT-PCR in NTHi-treated and untreated human cervix epithelial HeLa cells. HeLa cells transfected with human wild-type TLR2 expression plasmid (hTLR2) served as a positive control for TLR2 expression, whereas Cyclophilin was used as a control for amount of RNA used in each reaction. Reverse transcriptase (RT) was either included or not included in RT reactions to assure that the TLR2 PCR products result from cDNA, but not genomic DNA. RT-PCR analysis was carried out in duplicate. (B) Real-time quantitative PCR was performed to confirm up-regulation of TLR2 at mRNA level in HeLa, HMEEC-1 (human middle ear epithelial) and NHBE (primary human airway epithelial) cells. 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. (C) NTHi up-regulates TLR2 mRNA in HeLa cells in a time-dependent manner. TLR2 mRNA was measured by real-time quantitative PCR. Similar result was also observed in NHBE cells. (D) NTHi strongly up-regulates TLR2, but not TLR4 in HeLa cells, as assessed by real-time quantitative PCR. Similar result was also observed in NHBE cells. (E) NTHi also up-regulates expression of TLR2 at protein level as assessed by Western blot analysis. HeLa cells were treated with and without NTHi for 5 h. Protein lysates were then analyzed by Western blotting using anti-hTLR2 antibody (H-175). Equal amounts of proteins were loaded. HeLa cells transfected with human wild-type TLR2 expression plasmid (TLR2) served as a positive control for TLR2 expression. Similar result was also observed in NHBE cells. (F) The expression of human TLR2 is higher in mucosae from chronic otitis media patients in comparison to that from normal subjects. Immunohistochemical analysis of human TLR2 expression was performed in human middle ear mucosae from chronic otitis media patients and normal subjects. Archival human temporal bone sections were stained with a polyclonal anti-human TLR2 antibody.
(H-175). Note that the middle ear epithelium is thickened in the inflamed middle ear mucosa from chronic otitis media patients. Bar, 50 µm.

**Figure 2.** IKKβ-ικBα-dependent translocation and activation of NF-κB is required for NTHi-induced TLR2 up-regulation. (A) CAPE, MG-132 and overexpression of a transdominant mutant of ικBα inhibit NTHi-induced TLR2 up-regulation in HeLa cells. Similar results were also observed in NHBE cells. (B and C) Concomitantly, NTHi-induced NF-κB activation and translocation was also blocked by MG-132 in HeLa cells. Similar result was also observed in NHBE cells. (D) Overexpression of a dominant-negative mutant form of IKKβ [IKKβ(K44A)]abrogated NTHi-induced TLR2 up-regulation in HeLa cells. Similar results were also observed in NHBE cells. (E and F) Overexpression of wild-type p65 induces TLR2 mRNA and NF-κB activation in a dose-dependent manner in HeLa cells. All real-time quantitative PCR reactions were carried out in duplicate. Values are the mean ± SD; n=3.

**Figure 3.** Activation of MKK3/6-p38 α/β MAP kinase pathway is negatively involved in NTHi-induced TLR2 expression. (A) SB203580 greatly enhances NTHi-induced TLR2 up-regulation in HeLa cells in a dose-dependent manner. (B) Overexpression of a dominant-negative mutant form of either p38α [fp38α(AF)] or p38β [fp38β(AF)] also enhances, whereas overexpression of a wild-type p38α or p38β reduces, the NTHi-induced TLR2 expression in HeLa cells. (C) Overexpression of a dominant-negative mutant form of either MKK3 [MKK3b(A)] or MKK6 [MKK6b(A)] enhances NTHi-induced TLR2 up-regulation in HeLa cells. All real-time quantitative PCR reactions were carried out in duplicate. Values are the mean ± SD; n=3. Similar results were also observed in NHBE cells.
Figure 4. Glucocorticoids synergistically enhance NTHi-induced TLR2 up-regulation via a likely negative cross-talk with p38 MAP kinase pathway. (A) Dexamethasone (DEX) synergistically enhances NTHi-induced TLR2 up-regulation at mRNA level in HeLa cells in a dose-dependent manner. Similar results were also observed in NHBE and HMEEC-1 cells. (B) Dexamethasone (DEX) (1 µM) also synergistically enhances NTHi-induced TLR2 up-regulation at protein level in HeLa cells as assessed by Western blot analysis using anti-hTLR2 antibody (IMG-319). Equal amounts of proteins were loaded. Similar result was also observed in NHBE cells. (C) RU486 (1 µM) counteracts the enhancing effect of DEX (1 µM) on NTHi-induced TLR2 up-regulation at mRNA level in HeLa. (D) Overexpression of wild-type human GR (phGR) in HeLa cells further greatly enhances the synergistic effect of DEX on NTHi-induced TLR2 up-regulation. (E) Effects of DEX on NTHi-induced TLR2 up-regulation in HeLa cells that were pretreated with SB203580. (F) NTHi-induced phosphorylation of p38 MAP kinase is inhibited by DEX (1 µM) pretreatment in HeLa cells, which can be counteracted by RU486 (1 µM). GR, glucocorticoid receptor. All real-time quantitative PCR reactions were carried out in duplicate. Values are the mean ± SD; n=3.

Figure 5. Glucocorticoids attenuated NTHi-induced NF-κB activation. HeLa cells were transiently transfected with an NF-κB-regulated luciferase reporter construct and were then pretreated with 1 µM DEX for 2h before being stimulated with NTHi for 3h. Luciferase activity was then assessed in NTHi-treated and untreated cells. Values are the means ± SD; n = 3.

Figure 6. Schematic representation of the signaling pathways involved in TLR2 up-regulation by NTHi and glucocorticoids in human epithelial cells. As indicated, NTHi up-regulates TLR2 expression via a positive IKKβ-IκBα-dependent NF-κB pathway and a
likely negative MKK3/6-p38 α/β pathway. Glucocorticoids, well-known potent anti-inflammatory agents, synergistically enhance NTHi-induced TLR2 up-regulation likely via a negative cross-talk with the inhibitory p38 MAP kinase pathway. The up-regulated TLR2 leads to enhanced immune and inflammatory responses. GR, glucocorticoids receptor; COPD, chronic obstructive pulmonary disease.
Figure 1A

TLR2
Cyclophilin

| RT | + | + | - | - | + | + | - | - | + | + |
|----|---|---|---|---|---|---|---|---|---|---|
| CON|   |   |   |   |   |   |   |   |   |   |
| NTHi|   |   |   |   |   |   |   |   |   |   |
| hTLR2|   |   |   |   |   |   |   |   |   |   |
Figure 1B

![Bar graph showing the relative quantity of TLR2 mRNA in HeLa, HMEEC-1, and NHBE cells under control (CON) and NTHi conditions.](http://www.jbc.org/)

- **HeLa**
  - Control (CON): Low quantity
  - NTHi: High quantity
- **HMEEC-1**
  - Control (CON): Low quantity
  - NTHi: Moderate quantity
- **NHBE**
  - Control (CON): Low quantity
  - NTHi: Moderate quantity

The graph illustrates the relative mRNA levels for TLR2 under different conditions.
Figure 1C

Relative Quantity of TLR2 mRNA

CON

NTHi

CON  1  1.5  2  3  5

(h)
Figure 1D

The figure shows a bar graph comparing the relative quantity of TLR mRNA for TLR2 and TLR4 under CON and NTHi conditions. The y-axis represents the relative quantity of TLR mRNA, ranging from 0 to 9. The x-axis distinguishes between TLR2 and TLR4. The bars indicate a significant increase in TLR2 mRNA under the NTHi condition compared to the CON condition, whereas TLR4 mRNA shows a much smaller increase.
Figure 1E
Figure 1F

Normal

Otitis Media

TLR2
Figure 2A
Figure 2B

Relative Luciferase Activity

CON  NTHi

CON  -  0.01  0.1  1  MG-132 (µM)

NTHi
Figure 2C

CON  NTHi  MG-132
Figure 2D

Graph showing relative quantity of TLR2 mRNA. The x-axis represents conditions including CON, IKKβ (K44A), and NTHi. The y-axis represents the relative quantity of TLR2 mRNA with values ranging from 0 to 6. The CON condition shows a lower relative quantity compared to the NTHi condition, which shows a significantly higher value, especially when treated with IKKβ (K44A).
Figure 2E
Figure 2F

![Graph showing relative luciferase activity](image)

- **Relative Luciferase Activity**
- **vector** and **p65**
- Dosing levels: 0.005, 0.01, 0.025, 0.05 µg
Figure 3A

![Graph showing relative quantity of TLR2 mRNA for CON and NTHi](image-url)

- **X-axis**: SB (0, 0.1 μM, 0.5 μM, 1 μM, 5 μM, 20 μM)
- **Y-axis**: Relative Quantity of TLR2 mRNA (0, 5, 10, 15, 20, 25, 30)

Legend:
- **CON** (white bars)
- **NTHi** (black bars)
Figure 3B

Relative Quantity of TLR2 mRNA

- CON
- NTHi

0 2 4 6 8 10 12

vector p38α(AF) p38β2(AF) p38α(WT) p38β2(WT)
Figure 3C

![Graph showing relative quantity of TLR2 mRNA]

- **CON**
- **NTHi**

**X-axis:**
- Vector
- MKK3b(A)
- MKK6b(A)

**Y-axis:** Relative Quantity of TLR2 mRNA
Figure 4A
Figure 4B
Figure 4C

[Bar graph showing relative quantity of TLR2 mRNA for different treatments: NTHi, DEX, RU486.]
Figure 4D
Figure 4E

Relative Quantity of TLR2 mRNA

---

CON
NTHi

---

DEX (0.1 µM) - + - +
SB (5 µM) - - + +

---

DEX (1 µM) - + - +
SB (5 µM) - - + +

---

DEX (0.1 µM) - + - +
SB (0.1 µM) - - + +
Figure 4F

|       | pp38 | p38  |
|-------|------|------|
| NTHi  | -    | +    | -    | +    | +    | +    |
| DEX   | -    | -    | +    | +    | +    |      |
| RU486 | -    | -    | -    | -    | -    | +    |
Figure 5

![Bar graph showing relative luciferase activity with DEX at different concentrations and CON control. The graph compares CON and NTHi conditions.](image-url)
Figure 6

NTHi

IKKβ

IKKβ

MKK3/6

p38α/β2

NF-κB

IKBα

NF-κB

Gr

TLR2

? P

P

P

P

? ±

Immune/Inflammatory Responses in Otitis Media and COPD

TLR2 Gene Expression
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