Critical Review

Exploitation of Host Epithelial Signaling Networks by Respiratory Bacterial Pathogens

Jian-Dong Li^{1,2,*}¹Gonda Department of Cell and Molecular Biology, House Ear Institute,
2100 West Third Street, Los Angeles, CA 90057, USA²Department of Otolaryngology, University of Southern California, Los Angeles, CA 90057, USA

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Abstract. Although tremendous effort has been put towards identifying the surface molecules of nontypeable *Haemophilus influenzae* (NTHi) for vaccine development over the past decades, it is only recently that we have begun to appreciate the intricate host epithelial signaling networks activated by NTHi, an important human pathogen causing respiratory infections. From what has been reported, it is evident that NTHi activates multiple signaling pathways in host epithelial cells that, in turn, inadvertently contribute to the pathogenesis. Among those signaling pathways, activation of NF- κ B leads to up-regulation of IL-1 β , IL-8 and TNF- α , mucin *MUC2* and Toll-like receptor 2 (TLR2), whereas activation of p38 MAP kinase mediates not only up-regulation of inflammatory mediators and mucin *MUC5AC* but also down-regulation of TLR2. Interestingly, NTHi-induced activation of the PI3K-Akt pathway, however, leads to inhibition of p38 mitogen-activated protein (MAP) kinase. Moreover, the TGF- β -Smad signaling pathway co-operates with NF- κ B to mediate up-regulation of mucin *MUC2*. Finally, glucocorticoids synergistically enhance NTHi-induced TLR2 expression via specific up-regulation of the MAP kinase phosphatase-1 that, in turn, leads to inactivation of p38 MAP kinase, the negative regulator for TLR2 expression. These studies may bring new insights into the molecular pathogenesis of NTHi-induced infections and open up novel therapeutic targets for these diseases.

Keywords: chronic obstructive pulmonary disease, glucocorticoids, p38 MAPK, nuclear factor- κ B, nontypeable *Haemophilus influenzae*

Introduction

Recently, interest in exploitation of host cell signaling machinery by bacteria has increased because of the dramatic increase in antibiotic-resistant strains, the emergence of new pathogens and the resurgence of old ones. Bacterial pathogens have evolved many sophisticated ways to subvert normal host cellular responses. One such subversive pathogen is the gram-negative bacterium nontypeable *Haemophilus influenzae* (NTHi), which exemplifies an important, as yet underexplored, human respiratory pathogen in both children and adults (1). In children, it causes otitis media (OM), the most

common childhood infection and the leading cause of conductive hearing loss (2, 3), while in adults, it exacerbates chronic obstructive pulmonary diseases (COPD), the fourth leading cause of death in the United States (4, 5). Despite the need for prophylactic measures, development of a vaccine for preventing NTHi infections has been difficult and still remains a great challenge (1). Moreover, inappropriate antibiotic treatment contributes to the worldwide emergence of antibiotic-resistant strains of NTHi. Therefore, alternative therapeutic strategies are urgently needed to counter NTHi infections that are previously treated by conventional antibiotics. Development of these novel therapeutic reagents, however, requires a full understanding of the molecular mechanisms, especially the signaling mechanisms underlying the NTHi infections.

*Corresponding author (affiliation #1). FAX: +1-213-273-8088
E-mail: jdli@hei.org

NF- κ B plays a critical role in NTHi-induced inflammation and up-regulation of mucin, hallmarks of COPD and otitis media

Although much work has been done on identifying the surface molecules of NTHi for vaccine development (1, 2), the molecular mechanisms including the signaling mechanisms underlying the pathogenesis of NTHi infections still remain poorly understood. Like most other gram-negative bacterial infections, both otitis media and chronic obstructive pulmonary diseases are characterized by inflammation, which is mainly mediated by inflammatory cytokines and chemokines such as interleukin-1 β (IL-1 β), interleukin-8 (IL-8) and tumor necrosis factor- α (TNF- α) (6–8). Among a variety of transcription regulators, nuclear factor κ B (NF- κ B) has been shown to play a critical role in regulating the expression of large numbers of genes including cytokines, chemokines and other mediators involved in inflammatory responses (9).

NF- κ B is a dimeric transcription factor that is composed of p50 (NF- κ B1) and p65 (RelA) subunits (9). In resting cells, NF- κ B is retained in the cytoplasm but enters the nucleus in response to various stimuli including bacterial infections. Activation of NF- κ B is controlled by an inhibitory subunit, I κ B, which retains NF- κ B in the cytoplasm. The activation of NF- κ B requires sequential phosphorylation, ubiquitination, and degradation of I κ B as well as consequent exposure of a nuclear localization signal on NF- κ B molecule. Multiple kinases have been shown to phosphorylate I κ B at specific amino-terminal serine residues (10, 11). The most well studied kinases are I κ B kinases, IKK α (or IKK-1) and IKK β (or IKK-2) (9). There is also strong evidence that IKK α and IKK β are themselves phosphorylated and activated by one or more upstream activating kinases. One such upstream kinase, NF- κ B inducing kinase (NIK), was recently identified (9). Phosphorylation of I κ B by the IKKs pathway will eventually lead to the nuclear translocation of NF- κ B, which, in turn, activates expression of target genes in the nucleus.

Based on the essential involvement of NF- κ B in inflammatory responses, activation of NF- κ B by NTHi has been recently fully explored. Interestingly, NTHi appears to strongly activate NF- κ B in human epithelial cells via two distinct signaling pathways, NF- κ B translocation-dependent and independent pathways (12). The NF- κ B translocation-dependent pathway involves activation of the NIK-IKK α / β complex, leading to I κ B α phosphorylation and degradation, while the NF- κ B translocation-independent pathway involves activation of the MKK3/6-p38 MAP kinase pathway. Bifurcation of NTHi-induced NIK-IKK α / β -I κ B α and MKK3/6-

p38 MAP kinase pathways may occur at TGF- β -activated kinase I (TAK1). Furthermore, Toll-like receptor 2 (TLR2) is required for NTHi-induced NF- κ B activation. In addition, several key inflammatory mediators including IL-1 β , IL-8 and TNF- α are up-regulated by NTHi. Finally, P6, a 16-kDa lipoprotein highly conserved in the outer membrane of all NTHi and *H. influenzae* type b strains, appears to also activate NF- κ B via similar signaling pathways. Taken together, these results demonstrate that NTHi activates NF- κ B via a TLR2-TAK1-dependent NIK-IKK α / β -I κ B α and MKK3/6-p38 MAP kinase signaling pathways that, in turn, leads to up-regulation of key inflammatory mediators (Fig. 1). In addition to its role in regulating inflammatory responses, activation of NF- κ B via a NIK-IKK α / β -I κ B α signaling pathway has been also shown to mediate NTHi-induced up-regulation of mucin *MUC2* and TLR2 (13, 14). These studies may lead to new therapeutic intervention for NTHi-induced infections including otitis media and chronic obstructive pulmonary diseases.

p38 MAP kinase also plays a key role in the pathogenesis of NTHi infections

In addition to NF- κ B pathway p38 MAP kinase (mitogen-activated protein kinase) (15, 16) represents another key signaling module involved in mediating cellular stress responses during bacterial infections. p38 MAP kinase consists of 4 isoforms, α , β , γ , and δ . Together with its upstream MAP kinase kinases (MKKs), p38 MAP kinase comprises another important MAP kinase pathway (16). The p38 MAP kinase pathway is in many ways homologous to the classical extracellular signal-regulated kinases 1 and 2 (ERK1/2) pathway, but it appears to be activated by distinct upstream kinases, MKK3, MKK4 and MKK6, and is stimulated by a variety of cellular stresses including osmotic shock, inflammatory cytokines and LPS (17). Activated p38 MAP kinase, either directly activates transcription factors such as TCF, ATF2, and Max, or it activates downstream protein kinases such as p38-regulated/activated protein kinase (PRAK), which, in turn, activates the transcription factors (18, 19). Development of p38 MAP kinase inhibitors provides a powerful tool for studying the role of p38 in stress-induced cellular responses. Among the inhibitors, SB203580 has been shown to selectively inhibit p38 α and β , but not γ or δ -nor any other member of the MAP kinase family (20, 21). Because of its high selectivity, SB203580 has been widely used in cellular assays as a probe for the involvement of p38 (22). Moreover, tremendous effort is being put towards developing novel therapeutic agents that are based on the structure of SB203580 (22). Taking

advantage of SB203580 and available dominant-negative mutant constructs of p38, it has been shown recently that activation of p38 MAP kinase (α or β) is required for NTHi-induced *MUC5AC* transcriptional activation (23) (Fig. 2). In sharp contrast to its positive involvement in NTHi-induced mucin *MUC5AC* transcription, the MKK3/6-p38 α/β signaling pathway, however, serves as a negative regulator for NTHi-induced TLR2 transcription (14). These results thus indicate that NTHi also strongly activates p38 MAP kinase in addition to NF- κ B, which, in turn, leads to differential transcriptional regulation of host defense genes in the pathogenesis of NTHi infections.

Phosphoinositide 3-kinase (PI3-kinase)-Akt signaling pathway acts as a negative regulator for p38 MAP kinase

Besides NF- κ B and p38 MAP kinase pathways, phosphoinositide 3-kinase (PI3-kinase) represents another major signaling transducer involved in a variety of cellular responses. It is a heterodimer consisting of p85, the regulatory subunit, and p110, the catalytic subunit. Activation of PI3-kinase catalyzes the phosphorylation of phosphatidylinositol. The phosphorylated lipids bind to Akt, a serine-threonine kinase, resulting in membrane localization and a conformational change of Akt. This allows Akt to be phosphorylated and activated to mediate a variety of cellular responses such as protection of cells from apoptosis (24) and induction of NF- κ B (25). There is also evidence that PI3-kinase is involved in bacterial pathogenesis (26). Because of the importance of PI3-kinase-Akt in cellular responses as well as in bacterial pathogenesis, efforts have been put towards determining the potential involvement of PI3-kinase-Akt in NTHi-induced *MUC5AC* transcription. Interestingly, the PI3-kinase-Akt signaling pathway also appears to be activated by NTHi, which, however, leads to down-regulation of p38 MAP kinase activity (23) (Fig. 3). Negative cross-talk has been established by previous studies between the PI3-kinase-Akt pathway and MAP kinases including ERK and the c-jun NH₂-terminal kinase (JNK) (27). Whether or not there is also negative interaction between PI3-kinase-Akt and p38 MAP kinase has remained unclear. Recently, a report by Gratton et al. showed that blockade of PI3-kinase-Akt led to enhanced vascular endothelial growth factor (VEGF) activation of p38 MAP kinase (28). However, little is known about the involvement of this negative cross-talk in bacterial pathogenesis as well as in mucin gene regulation. In the case of NTHi infections, PI3-kinase-Akt serves as an inhibitory signaling pathway in NTHi-induced *MUC5AC* transcription via a

negative cross-talk with p38 MAP kinase. Although it has been shown that inhibition of the PI3-kinase-Akt signaling by wortmannin enhanced, whereas activation of PI3-kinase-Akt by overexpression of an activated form of p110 attenuated, NTHi-induced activation of p38 MAP kinase, it is difficult to rule out the possibility that the PI3-kinase-Akt pathway may interact with the upstream p38 MAP kinases such as MAP kinase kinase 3 and 6 (MKK3/6) based on current report. It is also unclear whether a direct physical interaction between PI3-kinase-Akt and MKK3/6-p38 MAP kinase is involved in this cross talk. These questions will be addressed in our future studies.

TGF- β signaling pathway cooperates with NF- κ B to mediate NTHi-induced host defensive response

The TGF- β -Smad pathway represents another important signaling pathway participating in regulation of diverse biological processes, including cell proliferation, differentiation, death, and inflammatory and immune responses (29–35). The TGF- β superfamily is a large group of secreted growth factors of which three subgroups have been defined: the TGF- β s, activins and bone morphogenetic proteins (BMPs) (32, 34). TGF- β initiates signaling through the ligand-dependent activation of a heteromeric complex of type II and type I receptors. The type II receptor kinase then phosphorylates the type I receptor in a conserved glycine-serine domain (GS domain), resulting in activation of the type I receptor. The activated type I receptor subsequently recognizes and phosphorylates the Smad subgroup known as receptor-activated Smads (R-Smad), including Smad 2 and 3. This causes dissociation of R-Smad from the receptor, stimulates the assembly of a heteromeric complex between the phosphorylated R-Smad and the Co-Smad, Smad 4, and then induces the translocation of the Smad complex to the nucleus, where the Smad complex regulates the expression of target genes (30, 32). In addition to its direct interaction with Smad DNA-binding element (SBE), growing evidence suggests that Smads also regulate gene transcription by direct interaction and functional cooperation with other transcription factors, such as NF- κ B (36–38). Despite its important role in regulation of diverse biological processes, it is still unclear if activation of the TGF- β -Smad signaling pathway also mediates up-regulation of mucin, a primary host innate defensive response to bacteria.

Because of the important role of NF- κ B and TGF- β signaling in mediating diverse cellular responses as well as the reported functional cooperation between NF- κ B and TGF- β -Smad, it is logical that the TGF- β -Smad signaling pathway cooperates with NF- κ B to mediate

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

up-regulation of *MUC2* mucin transcription in response to NTHi infections in human epithelial cells. Indeed, activation of TGF- β receptor-Smad3/4 signaling, together with TLR2-MyD88-TAK1-NIK-IKK β / γ -I κ B α -dependent activation of NF- κ B, has been recently shown to mediate NTHi-induced *MUC2* mucin transcription (13). These findings provided direct evidence, for the first time, that the bacterium NTHi uses TGF- β -Smad pathway for transducing signals into nucleus, at least in part, via an autocrine-independent mechanism, and that the functional cooperation between NF- κ B and TGF- β receptor-Smad is required for host defensive response to NTHi (Fig. 4). These studies may bring insights into the novel role of TGF- β -Smad signaling in bacterial pathogenesis and may lead to novel therapeutic intervention for otitis media and COPD.

Orchestration and optimization of host immune and defense responses by glucocorticoids in NTHi infections

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 (39 – 43). Moreover, the molecular basis for the beneficial role of glucocorticoids in the treatment of bacterial infections such as *Haemophilus influenzae*-induced bacterial meningitis also remains unclear. Recently, it was reported that glucocorticoids synergistically en-

hance 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* (14, 44). Thus, it is likely that glucocorticoids may not only suppress but also enhance the host immune and defense response. In addition, our study may also provide a 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. However, the signaling mechanisms underlying glucocorticoid-mediated synergistic enhancement of TLR2 are still unknown. Nor is the physiological relevance of TLR2 up-regulation known at present. Very recently, we showed that, glucocorticoids synergistically enhance the NTHi-induced TLR2 expression via specific up-regulation of the MAP kinase phosphatase-1 (MKP-1) (45 – 47), which, in turn, leads to dephos-

Fig. 1. Schematic representation of NTHi-induced signal transduction pathways involved in NF- κ B activation in human epithelial cells. IKK, I κ B kinase; NTHi, nontypeable *Haemophilus influenzae*; MKK, MAP (mitogen-activated protein) kinase kinase; TAK1, TGF- β -activated kinase 1; TLR, Toll-like receptor.

Fig. 2. Schematic representation of NTHi-induced signal transduction pathways involved in p38 MAP kinase activation in human epithelial cells. IKK, I κ B kinase; NTHi, nontypeable *Haemophilus influenzae*; MKK, MAP (kinase, mitogen-activated protein) kinase kinase; TAK1, TGF- β -activated kinase 1; TLR, Toll-like receptor.

Fig. 3. Schematic representation of NTHi-induced PI3-kinase-Akt signaling pathways involved in inhibition of p38 MAP kinase. IKK, I κ B kinase; NTHi, nontypeable *Haemophilus influenzae*; MKK, MAP (mitogen-activated protein) kinase kinase; PI3K: phosphoinositide 3-kinase; TAK1, TGF- β -activated kinase 1; TLR, Toll-like receptor.

Fig. 4. Schematic diagram showing that TGF- β signaling pathway cooperates with NF- κ B to mediate NTHi-induced host defensive response. IKK, I κ B kinase; NTHi, nontypeable *Haemophilus influenzae*; MKK, MAP (mitogen-activated protein) kinase kinase; TAK1, TGF- β -activated kinase 1; TGF- β R: transforming growth factor- β -receptor; TLR, Toll-like receptor.

Fig. 5. 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. DEX, dexamethasone; GR, glucocorticoids receptor; IKK, I κ B kinase; NTHi, nontypeable *Haemophilus influenzae*; MKK, MAP (mitogen-activated protein) kinase kinase; MKP-1, MAP kinase phosphatase-1; TAK1, TGF- β activated kinase 1; TLR, Toll-like receptor.

Fig. 6. Schematic representation of NTHi-induced host epithelial signaling networks in the pathogenesis of NTHi infections. DEX, dexamethasone; GR, glucocorticoids receptor; NTHi, nontypeable *Haemophilus influenzae*; MKK, MAP (mitogen-activated protein) kinase kinase; MKP-1, MAP kinase phosphatase-1; TAK1, TGF- β -activated kinase 1; TGF- β R: transforming growth factor- β receptor; TLR, Toll-like receptor.

phorylation and inactivation of p38 MAP kinase, the negative regulator for TLR2 expression (Fig. 5). Moreover, increased expression of TLR2 in epithelial cells also greatly enhance the NTHi-induced expression of several key cytokines including TNF- α , IL-1 β 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.

Concluding remarks

Although tremendous effort has been put towards identifying the surface molecules of NTHi for vaccine development over the past decades (1, 2), it is only recently that we have begun to appreciate the intricate host epithelial signaling networks activated by NTHi, an important human bacterial pathogen causing respiratory infections. From the studies that we have reviewed above, it is evident that NTHi activates multiple signaling pathways in host epithelial cells that, in turn, inadvertently contribute to the pathogenesis and disease outcome (Fig. 6). Among those signaling pathways activated by NTHi, activation of NF- κ B via a TLR2-TAK1-dependent NIK-IKK α / β -I κ B α leads to up-regulation of key inflammatory mediators including IL-1 β , IL-8 and TNF- α , mucin *MUC2* and TLR2 (13, 14), whereas activation of the MKK3/6-p38 MAP kinase signaling pathway mediates not only up-regulation of inflammatory mediators and mucin *MUC5AC* but also down-regulation of TLR2. Interestingly, NTHi-induced activation of PI3K-Akt pathway, however, leads to negative regulation of p38 MAPK activity. Moreover, a functional cooperation between the TGF- β -Smad signaling pathway and NF- κ B appears to mediate up-regulation of *MUC2* mucin transcription in response to NTHi infections. Finally, glucocorticoids synergistically enhance NTHi-induced TLR2 expression via specific up-regulation of MKP-1 that, in turn, leads to dephosphorylation and inactivation of p38 MAP kinase, the negative regulator for TLR2 expression. This result, although rather unexpected, may provide novel insights into the role of glucocorticoids in orchestrating and optimizing host defense and innate immune responses. These studies may bring new insights into molecular pathogenesis of NTHi-induced infections and open up new therapeutic targets for these diseases. Future efforts will be focused on identifying the key therapeutic targets for the treatment of NTHi infections using a multidisciplinary

approach including bacterial genetics and biochemistry, mammalian cell biology, molecular biology, immunology, and knockout mice.

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