Transforming Growth Factor-β-Smad Signaling Pathway Negatively Regulates Nontypeable Haemophilus influenzae-induced MUC5AC Mucin Transcription via Mitogen-activated Protein Kinase (MAPK) Phosphatase-1-dependent Inhibition of p38 MAPK*

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In contrast to the extensive studies on the role of transforming growth factor-β (TGF-β) in regulating cell proliferation, differentiation, and apoptosis over the past decade, relatively little is known about the exact role of TGF-β signaling in regulating host response in infectious diseases. Most of the recent studies have suggested that TGF-β inhibits macrophage activation during infectious interactions with pathogens such as Trypanosoma cruzi and Leishmania, thereby favoring virulence. In certain situations, however, there is also evidence that TGF-β has been correlated with enhanced resistance to microbes such as Candida albicans, thus benefiting the host. Despite these distinct observations that mainly focused on macrophages, little is known about how TGF-β regulates host primary innate defensive responses, such as up-regulation of mucin, in the airway epithelial cells. Moreover, how the TGF-β-Smad signaling pathway negatively regulates p38 mitogen-activated protein kinase (MAPK), a key pathway mediating host response to bacteria, still remains largely unknown. Here we show that nontypeable Haemophilus influenzae, a major human bacterial pathogen of otitis media and chronic obstructive pulmonary diseases, strongly induces up-regulation of MUC5AC mucin via activation of the Toll-like receptor 2-MycD88-dependent p38 pathway. Activation of TGF-β-Smad signaling, however, leads to down-regulation of p38 by inducing MAPK phosphatase-1, thereby acting as a negative regulator for MUC5AC induction. These studies may bring new insights into the novel role of TGF-β signaling in attenuating host primary innate defensive responses and enhance our understanding of the signaling mechanism underlying the cross-talk between TGF-β-Smad signaling pathway and the p38 MAPK pathway.

The TGF-β1 pathway represents a key signaling pathway participating in regulation of diverse biological processes (1–7). In contrast to the extensive studies on the role of TGF-β in regulating cell proliferation, differentiation, and apoptosis, relatively little is known about the exact role of TGF-β in regulating host response in infectious diseases. In review of the role of TGF-β signaling in infections, most studies have focused on pathogens that infect host macrophages such as Trypanosoma cruzi and a variety of Leishmania species (8). These studies have demonstrated that excessively produced TGF-β upon infection inhibits macrophage activation, thereby favoring virulence (9–11). In certain situations, however, there is also evidence that TGF-β has been correlated with enhanced resistance to microbes such as Candida albicans, thus benefiting the host (12). Despite these distinct observations that mainly focused on macrophages, little is known about how TGF-β regulates host innate defensive responses, such as up-regulation of mucin, in the mucosal epithelial cells of airways (13–17).

Mucins, the major component of mucous secretions, are high molecular weight and heavily glycosylated proteins synthesized by the mucosal epithelial cells lining the middle ear, trachea, and digestive and reproductive tracts (12–17). They protect the epithelial surface by binding and trapping inhaled infectious particles, including bacteria and viruses, for mucociliary clearance, at least in part because of the extraordinary diversity of their carbohydrate side chains (13, 14). Therefore, up-regulation of mucin in infectious diseases represents an important host innate defensive response to microbes (13). However, in patients with otitis media with effusion and chronic obstructive pulmonary diseases whose mucociliary clearance mechanisms have become defective, excessive production of mucin will lead to airway obstruction in chronic obstructive pulmonary disease and conductive hearing loss in otitis media with effusion (18–24). To date, 18 mucin genes have been identified (14–17). Among these, at least MUC2, MUC5AC, and MUC5B have been shown to play an important role in the pathogenesis of respiratory infectious diseases (14, 15, 17–21). We have demonstrated recently that TGF-β-Smad

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** To whom correspondence should be addressed: House Ear Inst., 2100 West Third St., Los Angeles, CA 90057. E-mail: jdl@hei.org.

The abbreviations used are: TGF-β, transforming growth factor-β; MAPK, mitogen-activated protein kinase; NTHI, nontypeable H. influenzae; MKP-1, MAPK phosphatase-1; DN, dominant-negative; TL22, Toll-like receptor 2; TjRIII, TGF-β receptor type II; SBE, Smad-binding element.
negative signaling pathway cooperates with NF-κB to mediate nontypeable Haemophilus influenzae-induced MUC2 mucin transcription (24). Still unknown is whether or not the TGF-β-Smad signaling pathway regulates MUC5AC, another key member of the mucin superfamily, in a similar manner. Understanding how TGF-β signaling mediates up-regulation of MUC5AC mucin may not only bring new insights into the novel role of TGF-β signaling in attenuating host primary innate defensive responses and may also open up novel therapeutic targets for these diseases.

In addition to the TGF-β-Smad signaling pathway, p38 MAPK, consisting of four isoforms, α, β, γ, and δ, together with its upstream MAPK kinases (MKK3/6), comprises another important signaling pathway mediating diverse cellular responses (25). Recent studies have shown that p38 MAPK is not only regulated by its immediate upstream kinases such as MKK3/6 but is also regulated by other signaling pathways such as TGF-β signaling pathway (26, 27). In contrast to the relatively extensive studies on the positive regulation of p38 by TGF-β signaling, relatively little is known about the negative regulation of p38 MAPK by TGF-β signaling, especially in the pathogenesis of bacterial infectious diseases.

Because of the important role of TGF-β signaling in mediating diverse cellular responses and our recent observations showing the activation of TGF-β and p38 signaling by nontypeable H. influenzae (NTHi) as well as the reported interaction between TGF-β and p38 pathways, we hypothesized that the TGF-β-Smad signaling pathway interacts with p38 to mediate up-regulation of MUC5AC mucin in response to NTHi in human epithelial cells. Here, we showed that NTHi, a major human bacterial pathogen of otitis media and chronic obstructive pulmonary disease, strongly induces up-regulation of MUC5AC mucin (28–33), a primary innate defensive response for mammalian airways, via activation of multiple signaling pathways. The activation of the TLR2-MyD88-dependent p38 MAPK pathway is required for NTHi-induced MUC5AC transcription, whereas activation of TGF-β receptor-mediated signaling leads to down-regulation of p38 MAPK by inducing MAPK phosphatase-1 (MKP-1) expression, thereby acting as a negative regulator for MUC5AC induction. These studies bring new insights into the novel role of TGF-β signaling in attenuating host primary innate defensive responses and enhance our understanding of the negative cross-talks between the TGF-β-Smad and p38 MAPK signaling pathways.

**MATERIALS AND METHODS**

**Reagents—**SB203580, cycloheximide, and Ro-31-8220 were purchased from Calbiochem (La Jolla, CA). Recombinant human TGF-β1 and TGF-β neutralization antibody were purchased from R & D Systems.

**Bacterial Strains and Culture Conditions—**NTHi strain 12, a clinical isolate, was used in this study (21, 24, 33). Bacteria were grown on chocolate agar at 37 °C in an atmosphere of 5% CO2. 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 μg/ml). After overnight incubation, NTHi were centrifuged at 10,000 × g for 10 min, and the supernatant was discarded. The resulting pellet of NTHi was suspended in 10 ml of MAPK pathway is required for NTHi-induced pathways. The activation of the TLR2-MyD88-dependent p38 MAPK, consisting of four isoforms, α, β, γ, and δ, together with its upstream MAPK kinases (MKK3/6), comprises another important signaling pathway mediating diverse cellular responses (25). Recent studies have shown that p38 MAPK is not only regulated by its immediate upstream kinases such as MKK3/6 but is also regulated by other signaling pathways such as TGF-β signaling pathway (26, 27). In contrast to the relatively extensive studies on the positive regulation of p38 by TGF-β signaling, relatively little is known about the negative regulation of p38 MAPK by TGF-β signaling, especially in the pathogenesis of bacterial infectious diseases.

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**Cell Culture—**Human colon epithelial cell line HM3 was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen) (21, 24). Human cervix epithelial cell line HeLa and human middle ear epithelial cell line HMEC-1 were maintained as described (17, 28). Primary human airway epithelial (NHBE)
from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phosphorylation of p38 and TβRII were detected as described and following the manufacturer’s instructions (24, 33).

Immunofluorescent Staining—The cells were cultured on four-chamber microscope slides. After NTHi or TGF-β1 treatment, the cells were fixed in paraformaldehyde solution (4%) and incubated with mouse anti-Smad4 monoclonal antibody for 1 h (Santa Cruz Biotechnology, Inc.). Primary antibody was detected with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Inc.). The samples were viewed and photographed using a Zeiss Axiophot microscope.

RESULTS

TLR2-MyD88-dependent Activation of p38 MAPK Is Required for NTHi-induced Up-regulation of MUC5AC Mucin, a Primary Innate Defensive Response for Mammalian Airways—We have recently demonstrated that NTHi up-regulates MUC5AC mucin transcription via p38 MAPK in human epithelial cells (21). The signaling mechanisms underlying the NTHi-mediated activation of p38 MAPK that leads to up-regulation of MUC5AC, however, have yet to be defined. To investigate how p38 MAPK is regulated by NTHi, we first confirmed the effect of NTHi on MUC5AC transcription and the requirement for p38 MAPK in MUC5AC induction. As shown previously, NTHi up-regulated MUC5AC expression at mRNA level in human epithelial HeLa and HM3 cells (Fig 1A) as assessed by performing real time quantitative PCR analysis. In addition, NTHi-induced MUC5AC expression was also observed in human middle ear epithelial HMEEC cells as well as in primary human bronchial epithelial NHBE cells. Moreover, as shown in Fig. 1B, the pyridinyl imidazole SB203580, a highly specific inhibitor for p38 MAPK, greatly inhibited NTHi-induced MUC5AC expression. Consistent with this finding, SB203580 and overexpression of a dominant-negative mutant of p38α or p38β also inhibited NTHi-induced MUC5AC transcription as assessed using a human MUC5AC promoter-luciferase reporter construct (Fig. 1C), thus confirming the requirement for p38 MAPK in MUC5AC induction.

We next sought to determine the receptor-mediated signaling pathway upstream of p38 that mediates NTHi-induced MUC5AC transcription. Based on our recent finding that NTHi induces p38-dependent activation of NF-κB via TLR2 (24, 33), we therefore determined the involvement of TLR2 signaling in MUC5AC induction. Interestingly, co-transfection of HM3 cells with a dominant-negative mutant of TLR2 inhibited NTHi-induced MUC5AC transcription, whereas overexpression of a wild type TLR2 enhanced MUC5AC induction (Fig. 2A). Similarly, overexpressing a dominant-negative mutant MyD88, a key adapter protein downstream of TLR2 (38), also inhibited MUC5AC induction. Concomitantly, overexpression of dominant-negative mutants of TLR2 and MyD88 also abrogated MUC5AC induction at the mRNA level as assessed by performing real time quantitative PCR analysis (Fig. 2B). To address whether the TLR2-MyD88 signaling pathway also mediates NTHi-induced MUC5AC in primary human bronchial epithelial cells, we next studied the effects of overexpressing dominant-negative mutants of TLR2 and MyD88 on MUC5AC induction in NHBE cells. As shown in Fig. 2C, NTHi-induced MUC5AC expression at mRNA level was also abolished by these treatments in primary NHBE cells. These data indicate that the TLR2-MyD88 signaling pathway is required for MUC5AC induction.

To further determine whether TLR2-MyD88 acts upstream of p38 MAPK, we assessed the effect of overexpressing the same dominant-negative mutants of TLR2 and MyD88 on NTHi-induced p38 phosphorylation by using anti-phosphorylated p38 MAPK antibody. Indeed, co-transfecting the HM3 cells with both dominant-negative mutants inhibited NTHi-induced p38 phosphorylation (Fig. 2D). Thus, it is evident that the TLR2-MyD88 signaling pathway mediates NTHi-induced up-regulation of MUC5AC via activation of p38 MAPK.

TGF-β Type II Receptor-Smad3/4 Signaling Negatively Regulates NTHi-induced MUC5AC Transcription—Because of the important role TGF-β signaling plays in regulating host immune responses in bacterial infections and our recent study showing the positive involvement of TGF-β signaling in NTHi-induced MUC5AC transcription via p38 MAPK, we assessed the effect of overexpressing the TGF-β receptor Type II, a key adapter protein downstream of TLR2 (38), also inhibited NTHi-induced MUC5AC transcription. As we showed previously, NTHi-induced MUC5AC expression at mRNA level was also abolished by co-transfecting the HM3 cells with both dominant-negative mutants inhibited NTHi-induced up-regulation of MUC5AC transcrip-

FIG. 1. NTHi up-regulates human MUC5AC mucin transcription via p38 MAPK. A, NTHi-induced up-regulation of MUC5AC mucin expression at the mRNA level was observed in a variety of human epithelial cell lines including HeLa, HM3, and HMEEC-1 cells as well as primary human bronchial epithelial (NHBE) cells as assessed by performing real time quantitative PCR analysis. B, SB203580, a specific inhibitor for p38 MAPK, inhibited NTHi-induced MUC5AC up-regulation at mRNA level. C, effects of SB203580 and co-expressing a dominant-negative mutant of p38α or p38β on NTHi-induced MUC5AC transcription. MUC5AC TK-Luc construct was transfected or co-transfected with dominant-negative mutants of p38α and p38β into HM3 cells. Transfected cells were pretreated with or without SB203580 for 1 h. NTHi was then added to the transfected cells 42 h after transfection. After 5 h, the cells were harvested for luciferase assay. The values are the means ± S.D. (n = 3). CON, control.
induced transcription of MUC2 (8, 24), another key member of mucin superfamily, we were interested in determining whether TGF-β-receptor signaling also mediates NTHi-induced up-regulation of MUC5AC mucin, a primary innate defensive response for host respiratory mucosa (13). We first examined the effects of overexpressing the dominant-negative mutant and wild type of TβRII on NTHi-induced MUC5AC expression (40, 41). Surprisingly, co-transfecting the HM3 cells with a dominant-negative mutant of TβRII greatly enhanced NTHi-induced MUC5AC expression at the mRNA level, whereas overexpressing the wild type TβRII attenuated MUC5AC induction (Fig. 3A). We next confirmed the negative involvement of TβRII signaling in MUC5AC induction in primary human bronchial epithelial NHBE cells. As shown in Fig. 3B, co-transfecting NHBE cells with a dominant-negative TβRII also enhanced MUC5AC induction at the endogenous mRNA level. Thus, these data indicate that TβRII signaling negatively regulates NTHi-induced MUC5AC expression in human epithelial cells.

To further confirm whether TβRII signaling indeed acts as a negative regulator for MUC5AC induction, we took advantage of the available DR26 cell, a lung epithelial cell line that is derived from the wild type Mv1Lu cells and that lacks functional TβRII (24, 34). We first assessed the effect of TGF-β1 on SBE-dependent promoter activity in Mv1Lu and DR26 cells, respectively. As expected, no TGF-β1-induced promoter activity was observed in mutant DR26 cells, whereas the wild type Mv1Lu cells showed potent induction of SBE-dependent promoter activity by TGF-β1 (Fig. 3C, left panel). We next examined the effects of NTHi on MUC5AC promoter activity in the same cells, respectively. As shown in Fig. 3C (right panel), almost no MUC5AC induction by NTHi was observed in wild type Mv1Lu cells, whereas NTHi induced MUC5AC promoter activity in mutant DR26 cells by ~2.5-fold. In accordance with these results, co-transfecting the wild type Mv1Lu cells with a dominant-negative mutant TβRII abolished the TGF-β1-induced SBE response, whereas co-transfecting the mutant DR26 cells with a wild type TβRII rescued the SBE response to TGF-β1 (Fig. 3D, upper panels). In contrast to the SBE response to TGF-β1, overexpressing a dominant-negative mutant TβRII in wild type Mv1Lu cells rendered the cells MUC5AC-responsive to NTHi, whereas co-transfecting the mutant DR26 cells with a wild type TβRII abolished the MUC5AC response to NTHi (Fig. 3D, lower panels). Thus, these data suggest that TβR signaling is a negative regulator for NTHi-induced MUC5AC mucin transcription in human epithelial cells.

Because the negative involvement of TβR signaling in MUC5AC induction was determined mainly by using overexpression of TβR expression plasmids and the TβR mutant cell lines, we next sought to confirm whether NTHi indeed activates TGF-β signaling like TGF-β does. We first evaluated the effect of NTHi on phosphorylation of TβRII by using an antibody against phosphorylated TβRII. As shown in Fig. 3E (left panel), NTHi, like TGF-β1, induced phosphorylation of TβRII. We next investigated whether NTHi activates TGF-β-Smad signaling by evaluating NTHi-induced nuclear translocation of Smad4, a key step for the Smad3/4 complex to exert its transcriptional activity (2). Fig. 3E (right panel) shows that NTHi potently induces nuclear translocation of Smad4. As expected, Smad4 translocation was also induced by TGF-β1. To further confirm whether NTHi activates TGF-β-Smad-dependent transcriptional activity, we assessed the effect of NTHi on SBE-dependent promoter activity by using SBE luciferase reporter in
FIG. 3. TGF-β type II receptor-mediated signaling negatively regulates NTHi-induced MUC5AC transcription. A, overexpression of a dominant-negative mutant of TβRII enhances NTHi-induced MUC5AC up-regulation, whereas co-expression of a wild type TβRII inhibits MUC5AC induction at mRNA level, as assessed by performing real time quantitative PCR analysis in HM3 cells. B, overexpression of a dominant-negative mutant of TβRII also enhances NTHi-induced MUC5AC up-regulation in primary bronchial epithelial NHBE cells. C, NTHi induces MUC5AC transcription in mutant DR26 cells lacking functional TβRII but not in Mv1Lu cells expressing wild type TβRII. A MUC5AC-TK-Luc or SBE-Luc reporter vector was transfected into Mv1Lu cells or DR26 cells. NTHi or recombinant human TGF-β1 was then added to the transfected cells 42 h after transfection. After 5 h, the cells were harvested for luciferase assay. D, co-transfecting wild type Mv1Lu cells with a dominant-negative mutant of TβRII renders these cells responsive to NTHi, whereas overexpressing a wild type TβRII construct in DR26 cells abolishes their responsiveness to NTHi. Expression plasmid of wild type or dominant-negative TβRII was co-transfected, as marked. E, NTHi and TGF-β1 induce phosphorylation of TβRII in HM3 cells as assessed using an antibody against phosphorylated TβRII (Tyr336) (left panel). In addition, NTHi and TGF-β1 also induce nuclear translocation of Smad4 and Smad-regulated promoter activity of SBE-Luc in HM3 (right panel). Representative fields of Smad4 fluorescence are shown in HM3 cells that are treated with NTHi or TGF-β1 (1 ng/ml) for 45 min, respectively. F, pretreatment of NTHi lysates with the TGF-β neutralization antibody, but not with the control antibody, enhances its ability to induce the transcriptional activity of MUC5AC promoter in HM3 cells promoter. The values are the means ± S.D. (n = 3). CON, control.
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Having identified TLR2-MyD88-p38 MAPK as a positive pathway and TβRII-Smad3/4 as a negative pathway involved in NTHi-induced MUC5AC transcription, it is still unclear whether other TGF-β family members may be involved in mediating the negative regulation of NTHi-induced MUC5AC transcription in an autocrine-dependent manner.

Because of importance of Smads in transducing TGF-β receptor-mediated signals into the nucleus (5, 37, 39), we next sought to determine the involvement of Smad3, one of the key receptor-activated Smads, and Smad4, the Co-Smad (common partner Smad). As shown in Fig. 4A, overexpression of a dominant-negative mutant of either Smad3 or Smad4 enhanced NTHi-induced MUC5AC expression, whereas co-transfection with the wild type form of either Smad3 or Smad4 inhibited MUC5AC induction at the mRNA level as assessed by performing real time quantitative PCR analysis in HM3 cells. In addition, activation of TGF-β-Smad signaling by co-expression of wild type Smad3 and Smad4 markedly abrogated MUC5AC induction at the mRNA level (Fig. 4B). Similar to their inhibitory effect on MUC5AC induction at the mRNA level, co-expression of wild type Smad3 and Smad4 also abolished NTHi-induced MUC5AC transcription at the transcriptional level as evaluated by co-transfecting the HM3 cells with a MUC5AC promoter luciferase reporter construct (Fig. 4C). Collectively, these results demonstrated that TGF-β type II receptor-Smad3/4 signaling is negatively involved in NTHi-induced MUC5AC transcription, thereby inhibiting the primary innate defensive response in host airway mucosa.

TβRII-Smad3/4 Signaling Pathway Negatively Mediates NTHi-induced MUC5AC via a Negative Cross-talk with p38 MAPK—Having identified TLR2-MyD88-p38 MAPK as a positive pathway and TβRI-Smad3/4 as a negative pathway involved in NTHi-induced MUC5AC transcription, it is still unknown whether or not there is a negative cross-talk between these two signaling pathways. To test the hypothesis that TGF-β signaling negatively regulates NTHi-induced MUC5AC transcription via down-regulating the p38 MAPK activity, we first investigated the effect of overexpressing a dominant-negative mutant of TβRII on NTHi-induced p38 phosphorylation. As shown in Fig. 5A, NTHi-induced p38 phosphorylation was greatly enhanced in HM3 cells transfected with a dominant-negative mutant of TβRII. The enhanced p38 phosphorylation was observed in HM3 cells treated with NTHi for various times. The highest level of the enhanced p38 phosphorylation was observed at 60 min. In addition, the enhancement of NTHi-induced p38 phosphorylation by inhibition of TGF-β signaling were also observed in primary bronchial epithelial NHBE cells (Fig. 5B). Similarly, co-transfecting the HM3 cells with a dominant-negative mutant of either a Smad3 or Smad4 also markedly enhanced the NTHi-induced p38 phosphorylation (Fig. 5C). In agreement with these results, addition of exogenous TGF-β1 in parallel with NTHi attenuated NTHi-induced p38 phosphorylation in both HM3 and primary NHBE cells (Fig. 5, D and E). Moreover, exogenous TGF-β1 also inhibited NTHi-induced MUC5AC transcription in a dose-dependent manner (Fig. 5F). These results thus confirmed our hypothesis that...
TβR-Smad3/4 signaling indeed acts as a negative regulator for NTHi-induced MUC5AC transcription via inhibiting p38 activation.

TGF-β-Smad Signaling Negatively Regulates NTHi-induced MUC5AC Induction via a MAPK Phosphatase-1-dependent Inhibition of p38 MAPK—One key issue that has yet to be addressed is how TGF-β-Smad signaling down-regulates NTHi-induced p38 activation. We first determined the possible involvement of de novo protein synthesis in NTHi-induced MUC5AC expression at the mRNA level by using the protein synthesis inhibitor cycloheximide. As shown in Fig. 6A, NTHi-induced up-regulation of MUC5AC at the mRNA level was further enhanced in HM3 cells pretreated with cycloheximide, indicating that de novo protein synthesis is negatively involved NTHi-induced MUC5AC expression. Based on this finding and the evidence that TβR signaling down-regulates NTHi-induced p38 phosphorylation, it is logical that TβR-Smad3/4 signaling may be involved in up-regulation of an inhibitor for p38 MAPK.

In review of the known inhibitors for p38, MKP-1, a member of a novel class of dual specificity phosphatases collectively termed MAPK phosphatases, represents a key protein phosphatase that dephosphorylates and inactivates p38 MAPK (36, 42). To determine whether TβR-Smad3/4 signaling is involved in the NTHi-induced MKP-1 expression, we next evaluated the effect of overexpression of a dominant-negative mutant of TβRII on NTHi-induced MKP-1 expression at the mRNA level. Interestingly, NTHi greatly induced MKP-1 expression at the mRNA level in a time-dependent manner, and the MKP-1 induction was greatly inhibited by overexpressing a dominant-negative mutant of TβRII (Fig. 6B). A similar result was also observed in primary bronchial epithelial NHBE cells (data not shown). Therefore, TβR signaling appears to be involved in NTHi-induced MKP-1 expression, which in turn leads to down-regulation of p38-dependent MUC5AC transcription.

To confirm the negative involvement of MKP-1 in NTHi-induced MUC5AC up-regulation, we then assessed the effect of Ro-31-8220, a chemical inhibitor for MKP-1 expression (36), on MUC5AC induction by NTHi in HM3 cells. As expected, Ro-31-8220 indeed enhanced NTHi-induced up-regulation of MUC5AC in a dose-dependent manner (Fig. 5C). To further confirm whether MKP-1 is indeed negatively involved in MUC5AC induction, we next investigated the effects of overexpressing an antisense and a wild-type full-length MKP-1 construct on NTHi-induced MUC5AC expression (36, 42, 43). As shown in Fig. 6D, overexpression of the antisense MKP-1 construct enhanced MUC5AC induction, whereas co-expression of a wild-type MKP-1 attenuated MUC5AC induction. Taken together, our data demonstrated that TGF-β-Smad signaling is negatively involved in NTHi-induced MUC5AC induction via MKP-1-dependent inhibition of p38 MAPK.

DISCUSSION

In conclusion, our studies have demonstrated that NTHi, a major human bacterial pathogen of otitis media and chronic obstructive pulmonary disease (28–33), strongly induces up-regulation of MUC5AC mucin, a primary innate defensive response for mammalian airway (13), via activation of multiple signaling pathways (Fig. 7). The activation of TLR2-MyD88-dependent p38 MAPK pathway is required for NTHi-induced MUC5AC transcription, whereas activation of TGF-β receptor-Smad3/4 signaling, however, leads to down-regulation of p38 MAPK by inducing MKP-1 expression, thereby acting as a negative regulator for MUC5AC induction.

A major finding in this study is the experimental evidence for the negative regulation of host mucosal defensive response to bacterial pathogen by TGF-β signaling. In review of the role of TGF-β signaling in infectious diseases (8), most studies have focused on pathogens that infect host macrophages such as T. cruzi and a variety of Leishmania species. These studies have demonstrated that excessively produced TGF-β upon infection inhibits macrophage activation, thereby favoring virulence (9–11). In certain situations, however, there is also evidence that TGF-β has been correlated with enhanced resistance to microbes such as C. albicans (12), thus benefiting the host. Despite these distinct observations that mainly focused on macrophages, little is known about how TGF-β regulates host innate defensive responses, such as up-regulation of mucin (13), in the mucosal epithelial cells of airway. Therefore, our study may bring new insights into the novel role of TGF-β signaling in attenuating host primary innate defensive responses to respiratory bacterial pathogens and may open up novel therapeutic targets for treatment of airway infectious diseases.

Another interesting finding in this study is the negative
cross-talk between the TGF-β/H9252-Smad3/4 signaling pathway and the p38 MAPK pathway. Experimental evidence over the past few years has suggested that TGF-β/H9252-Smad pathway may signal through interactions with other signaling pathways such as p38 MAPK. Although most of these studies have demonstrated an important role of TGF-β signaling in activation of p38 MAPK (26, 27), the negative regulation of p38 MAPK by TGF-β/Smad signaling still remains largely unknown. In the present study, we provided first hand evidence that activation of TGF-β/H9252-Smad3/4 signaling by bacterium NTHi leads to attenuation of p38 MAPK via up-regulation of MKP-1 in human airway epithelial cells. These observations should further enhance our understanding of the signaling mechanisms underlying the functional cross-talk between the TGF-β-Smad signaling pathway and the p38 MAPK pathway.

Finally, interesting evidence was also provided for the possible involvement of NTHi-derived TGF-β-like factor in activation of the TGF-β-Smad signaling pathway, which in turn leads to the negative regulation of TGF-β-induced MUC5AC transcription. Several lines of evidence support this notion. First, the NTHi-induced TβRII phosphorylation was observed at as early as 5 min (24). Given such an early phosphorylation of TβRII, it is likely that the early phosphorylation of TβRII may occur as a result of direct activation of TβRII by NTHi rather than NTHi-induced TGF-β autocrine signaling. Although TβRII is generally known as a serine/threonine kinase, there is also evidence that TβRII can function as a dual specificity kinase, and tyrosine phosphorylation may have an important role in TβRI signaling (44). Thus, NTHi-induced tyrosine phosphorylation of TβRII at 5 min may be at least interpreted as a TβR-mediated early response to NTHi factors. Second, pretreatment of NTHi lysates with the TGF-β neutral-
ization antibody enhanced its ability to induce the transcriptional activity of MUC5AC promoter as compared with the NTHI lysates treated with control antibody. Finally, NTHI did not induce any detectable increase in three major TGFB family members, TGFB1, 2, and 3, in the conditioned medium of HM3 cells. Taken together, these data suggest that TGFB-Smad signaling pathway is likely activated by NTHI-derived TGFB-like factor via a mechanism independent of TGFB-1, 2, and 3 autocrine signaling in the negative regulation of NTHI-induced MUC5AC transcription. However, our data do not rule out the possible involvement of the latent TGFB-bs stored in the extracellular matrix that might be activated by NTHI and then cross-talk with TgfRII. In addition, it is still unclear whether other TGFB family members are involved in mediating the negative regulation of NTHI-induced MUC5AC transcription.

Future studies will focus on determining the molecular identity of NTHI-derived TGFB-like factors and whether NTHI also activates the latent TGFB stored in the extracellular matrix that in turn leads to the activation of TGFB-s signaling. In addition, how TGFB-s signaling up-regulates MKP-1 expression via a Smad3/4-dependent mechanism will be further explored using biochemical and genetic approaches. Finally, the role of MKP-1 in mediating the negative cross-talk between TGFB-Smad pathway and the p38 pathway in vivo will also be confirmed by using MKP-1 knockout mice. These studies should deepen our understanding of the role of TGFB-s signaling in regulating host innate defensive response to respiratory bacterial pathogens.

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Transforming Growth Factor-β-Smad Signaling Pathway Negatively Regulates Nontypeable Haemophilus influenzae-induced MUC5AC Mucin Transcription via Mitogen-activated Protein Kinase (MAPK) Phosphatase-1-dependent Inhibition of p38 MAPK

Hirofumi Jono, Haidong Xu, Hirofumi Kai, David J. Lim, Young S. Kim, Xin-Hua Feng and Jian-Dong Li

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