The Transforming Growth Factor-β-Smad3/4 Signaling Pathway Acts as a Positive Regulator for TLR2 Induction by Bacteria via a Dual Mechanism Involving Functional Cooperation with NF-κB and MAPK Phosphatase 1-dependent Negative Cross-talk with p38 MAPK*

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The transforming growth factor β (TGF-β) pathway represents an important signaling pathway involved in the regulation of diverse biological processes, including cell proliferation, differentiation, and apoptosis. Despite the known role of TGF-βR-mediated signaling in suppressing immune response, its role in regulating human Toll-like receptors (TLRs), key host defense receptors that recognize invading bacterial pathogens, however, remains unknown. Here, we show for the first time that TGF-βR-Smad3/4 signaling pathway acts as a positive regulator for TLR2 induction by bacterium nontypeable Hemophilus influenzae (NTHi) in vitro and in vivo. The positive regulation of TLR2 induction by TGF-βR is mediated via a dual mechanism involving distinct signaling pathways. One mechanism involves functional cooperation between the TGF-βR-Smad3/4 pathway and NF-κB pathway. Another involves MAP kinase phosphatase 1 (MKP-1)-dependent inhibition of p38 MAPK, a known negative regulator for TLR2 induction. Moreover, we showed that TβR-mediated signaling is probably activated by NTHi-derived TGF-β mimicry molecule via an autocrine-independent mechanism. Thus, our study provides new insights into the role of TGF-β signaling in positively regulating host defense response by tightly controlling the expression level of TLR2 during bacterial infections and may lead to new therapeutic strategies for modulating host defense and inflammatory response.

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2 The abbreviations used are: TGF-β, transforming growth factor-β; TGF-βR, transforming growth factor-β receptor; TLR, Toll-like receptor; NTHi, nontypeable H. influenzae; MAPK, mitogen-activated protein kinase; MKP-1, MAPK phosphatase 1; IL, interleukin; mIL, mouse interleukin; DN, dominant negative; WT, wild type; siRNA, small interfering RNA; TNF, tumor necrosis factor.
Positive Regulation of TLR2 by TGF-β Signaling

studies from knock-out mice showing decreased survival of TLR2-deficient mice after infection with Gram-positive Staphylococcus aureus (26). Furthermore, our recent study demonstrated that TLR2 also plays a key role in activating host immune and inflammatory response by surface lipoprotein from the Gram-negative bacterium nontypeable Hemophilus influenzae (NTHi), a major cause of otitis media and exacerbation of chronic obstructive pulmonary diseases (27–31). Thus, it is clear that TLR2 plays a crucial role in host defense against both Gram-positive and Gram-negative bacteria. However, TLRs have also been implicated in some immune mediated and inflammatory diseases due to excessive immune responses. To avoid detrimental immune and inflammatory response, the host immune system needs to constantly strike a balance between activation and inhibition of immune response (32, 33). One important way to achieve such a balance is to tightly regulate the expression level of TLRs. Given the important role that TGF-β signaling plays in mediating a variety of cellular responses, such as proliferation, differentiation, and suppression of immune responses, it is likely that TGF-β signaling may also be critically involved in regulating the host defense response by tightly controlling the expression of TLRs, the most important host defense receptors.

In the present study, we provide evidence for the first time that TGF-βR acts as a positive regulator for TLR2 induction by bacterium NTHi via a Smad3/4-dependent mechanism. We showed that TβR-mediated signaling is probably activated by an NTHi-derived TGF-β mimicry molecule via an autocrine-independent mechanism. Moreover, our data indicate that TGF-β receptor-Smad3/4 signaling pathways are positively involved in TLR2 induction by NTHi via a dual mechanism involving functional cooperation with the NF-κB pathway and MAPK phosphatase 1 (MKP-1)-dependent inhibition of p38 MAPK, a negative regulator for TLR2 induction. Finally, we confirmed the positive regulation of TLR2 by TβR-Smad3/4 signaling in vivo. Thus, our study provides new insights into the role of TGF-β signaling in positively regulating host defense response by tightly controlling the expression level of TLR2 during bacterial infections and may help to further develop new therapeutic strategies for these diseases.

MATERIALS AND METHODS

Reagents—SB431542 was purchased from Sigma. SB203580, cycloheximide, and Ro31-8220 were purchased from Calbiochem. Recombinant human TGF-β1 was purchased from R&D Systems.

Bacterial Strains and Culture Conditions—NTHi strain 12, a clinical isolate, was used in this study (34). 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 phosphate-buffered saline and sonicated. Subsequently, the lysate was collected and stored at −70 °C. Live bacteria of NTHi at a multiplicity of infection of 100:1 were used to treat the epithelial cells. NTHi lysates (15 μg/ml) were used to treat the cells for all of the other experiments. First, NTHi lysates because of the following reasons. First, NTHi has been shown to be highly fragile and has the tendency to autolyse. Its autolysis can be triggered in vivo under various conditions including antibiotic treatment. 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 (13–14, 27, 35). Wild-type mink Mv1Lu and two cell lines, R1B and DR26, that are derived from Mv1Lu and lack functional TβRI and TβRII, respectively, were maintained as described (28, 38). Human breast cancer cell line MDA-MB-468 cells (American Type Culture Collection) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen). All media received additions of 100 units/ml penicillin and 0.1 mg/ml streptomycin.

Real Time Quantitative PCR Analysis of TLR2 and TGF-βs—Total RNA was isolated by using TRizol® Reagent (Invitrogen) by following the manufacturer’s instruction. For the reverse transcription reaction, TaqMan reverse transcription reagents (Applied Biosystems) were used. Briefly, the reverse transcription reaction was performed for 60 min at 37 °C, followed by 60 min at 42 °C by using oligo(dT) and random hexamers. PCR amplification was performed by using TaqMan Universal Master Mix for TLR2 and mTLR2 or SYBR Green Universal Master Mix for PAI-1, TGF-βs, mouse TNF-α, and sml-L-β. In brief, reactions were performed in duplicate containing 2 μl of Master Mix, 1 μl of template cDNA, 100 nM primers, and 100 μM probe in a final volume of 12.5 μl, and they were analyzed in a 96-well optical reaction plate (Applied Biosystems). Probes for TaqMan include a fluorescent reporter dye, 6-carboxyfluorescein, on the 5′-end and labeled with a fluorescent quencher dye, 6-carboxytetramethylrhodamine, on the 3′-end to allow direct detection of the PCR product. Reactions were amplified and quantified by using an ABI 7700 sequence detector and the manufacturer’s corresponding software (Applied Biosystems). The relative quantities of mRNAs were obtained by using the comparative Ct method (for details, see Ref. 44) and was normalized by using predeveloped TaqMan assay reagent human cyclophilin or mouse glyceraldehyde-3-phosphate dehydrogenase as an endogenous control (Applied Biosystems). The primers and probes for TLR2 were as follows: forward primer, 5′-GGC CAG CAA ATT ACC TGT GTG-3′; reverse primer, 5′-AGG CGG ACA TCC TGA ACC T-3′; TaqMan probe, 5′-6-carboxyfluorescein-TCC ATC CCA TGT GCG CCC CAG-3′ (23, 24). The primers for TGF-βs were as follows: TGF-β1 forward primer, 5′-CGA GCC TGA GCC CGA CTA C-3′; TGF-β1 reverse primer, 5′-AGA TTT CGT TGT GGG TTT CCA-3′; TGF-β2 forward primer, 5′-CCA TTA AGT GGA GTT GCT GTA CGT-3′; TGF-β2 reverse primer, 5′-GTG CCT ATT CCA TAG CAA TAC AGA A-3′; TGF-β3 forward primer, 5′-CAA ATT CAA AGG CGT GGA CA-3′; TGF-β3 reverse primer, 5′-CTT GAG GCG CCC CAG AT-3′.

Plasmids and Transfections—The expression plasmids of TβRI DN, TβRII WT, TβRII DN, TβRII WT, Smad3 DN,
Smad3 WT, Smad4 DN, Smad4 WT, fp38α (AF), fp38β2 (AF), MKP-1 WT and MKP-1 AS, transdominant mutant IkBa, IKKβ DN, WT p65, and WT p50 were previously described (27–28, 30). The reporter constructs of NF-κB-luc, SBE-luc, and PAI-1-luc were also previously described (27, 28, 30, 35). The reporter construct of the human TLR2 gene was kindly provided by Dr. M. Rehli (36). All of the transient transfections were carried out in duplicate or triplicate using TransIT-LT1 reagent (Mirus, Madison, WI) following the manufacturer’s instructions (27, 28, 30, 35). In all co-transfections, an empty vector was used as a control.

RNA-mediated Interference—TßRI, TßRII, and MKP-1 small interfering RNA oligonucleotides were purchased from Dharmacon. The siRNA was transfected into HeLa cells using RNAfect transfection reagent (Qiagen) following the manufacturer’s instructions (16, 17, 37).

Western Blot Analysis—Antibodies against phospho-Smad3, phospho-p38, p38, phospho-MKK3/6, and MKK3 were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against TßRII, TßRII, Smad3, TLR2, and MKP-1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody against β-actin was purchased from Sigma.

Phosphorylation of p38 and MKK3/6 was detected as described and following the manufacturer’s instructions (27, 35).

Immunofluorescent Staining—Cells were cultured on 4-chamber microscope slides. After TGF-β1 or NTHi treatment, the cells were fixed in 3.7% paraformaldehyde solution, incubated with phospho-Smad3, Smad3, or Smad4 antibody for 1 h. Primary antibody was detected with fluorescein isothiocyanate-conjugated anti-rabbit or mouse IgG (Santa Cruz, CA). Samples were viewed and photographed using a Zeiss Axioshot microscope.

TGF-β1 Neutralization Assays—For neutralization assays, TGF-β1 or NTHi lysate was mixed with purified IgG or human anti-TGF-β1 antibody (R&D Systems) at the indicated concentrations and incubated for 1 h before the addition to cells (28). Cells were treated for 3 h and collected for real time quantitative PCR analysis.

Immunodepletion—For immunodepletion, 60 μg of NTHi lysates was incubated with control rabbit IgG antibody and Protein A-agarose (Santa Cruz Biotechnology) for 30 min at 4 °C. After centrifugation, supernatants were incubated with or without human anti-TGF-β1 antibody (Cell Signaling) for 1 h at 4 °C, followed by incubation with Protein A-agarose overnight. Supernatants were then tested for their effects on TLR2 transcriptions.

TGF-β ELISA—For TGF-βs ELISA, ELISA plates were coated with 150 μl of 0.01 M citrate buffer and 150 μl of cell conditioned medium overnight at 4 °C. After washing with 0.05% PBS-T, 200 μl of 10 mg/ml bovine serum albumin was added and incubated at room temperature for 1 h. Following this, 100 μl of human TGF-β antibodies (R&D Systems) diluted in 0.05% PBS-T was added and incubated for 1 h. Next, 100 μl of horseradish peroxidase-conjugated anti-mouse antibody was added for 1 h. Finally, 100 μl of TMB substrate buffer (Bio-Rad) was added for 30 min and color-developed in relation to the amount of TGF-βs present. The reaction was stopped by adding 40 μl of 4 m sulfuric acid, and the degree of color that had been generated was determined by measuring the optical density at 450 nm.

In Vivo Study—BALB/c mice were purchased from Charles River Laboratories, Tlr2−/− mice were kindly provided by Dr. Shizuo Akira (Osaka University) (26), and 7–8-week-old mice were used in this study. After the trachea was surgically exposed by a middle line incision in the skin, NTHi was directly injected into the trachea. Lung tissues were collected and then stored at −80 °C. In experiments using chemical inhibitors, SB431542 (0.2 mg/mouse; 10 mg/kg) or Ro31-8220 (0.2 mg/mouse; 10 mg/kg) or an equal volume of control vehicle was given via an intraperitoneal route 30 min before the inoculation of NTHi into the trachea. Total RNA was isolated from the frozen tissue using TRIzol reagent. Three mice were used for each inoculation group. For in vivo bacterial clearance experiments, WT and Tlr2−/− mice were intratracheally inoculated with 50 μl of live bacterial suspension (1 × 10⁸ colony-forming units) for 6 h. To examine the effect of perturbing TGF-β signaling on bacteria clearance in mice, mice were pretreated with the TGF-βRI inhibitor SB431542 (10 mg/kg, intraperitoneally) 2 h before live NTHi inoculation. Six hours after bacteria inoculation, mice were sacrificed for bacterial clearance examination. The lung was removed, weighed, and homogenized in 1.5 ml of sterile PBS. One hundred microliters of homogenate and further serial 10-fold dilutions were plated on chocolate agar plates and incubated for 48 h at 37 °C in 5% CO₂. All animal experiments were approved by the Institutional Animal Care and Use Committee at the House Ear Institute and University of Rochester.

RESULTS

TGF-β Receptor I/II Smad3/4 Signaling Pathway Is a Positive Regulator for Bacterium NTHi-induced TLR2 Transcription—Despite the key role that TGF-β signaling plays in regulating diverse cellular responses, such as proliferation, differentiation, migration, and apoptosis as well as suppressing immune responses, its role in regulating human TLR in host defense response remains unknown (1–7). To determine whether TGF-β signaling is involved in bacterium-induced transcription of TLR2, a major member of TLR family (19–23), we assessed the effects of perturbing TGF-β signaling on bacterium NTHi-induced TLR2 transcription in human epithelial HeLa cells. As shown in Fig. 1A, SB431542, a specific inhibitor for TßRI (41), overexpressing a dominant negative mutant of TßRI, inhibited NTHi-induced TLR2 up-regulation, whereas overexpression of a wild-type TßRI enhanced it. Moreover, overexpression of a dominant negative mutant of TßRII also inhibited NTHi-induced TLR2 up-regulation, whereas overexpression of a wild-type TßRII enhanced it (Fig. 1B). These data suggest that activation of TßRII may be positively involved in NTHi-induced TLR2 up-regulation. We next confirmed the requirement of TßRII in TLR2 induction using a siRNA approach. We first examined the efficiency of TßRI or II-specific small interfering RNA (TßRI siRNA or TßRII siRNA) in reducing TßRI or II expression in HeLa cells. As expected, both TßRI and TßRII protein were markedly reduced by TßRI siRNA and TßRII siRNA, respectively (Fig. 1, C and D, right). We then assessed the effect of TßRI or II siRNA on TLR2 induction by NTHi. As shown in Fig. 1, C and D (left), TßRI and
Positive Regulation of TLR2 by TGF-β Signaling

**FIGURE 1.** TGF-β receptor I/II-mediated signaling pathway is a positive regulator for bacterium NTHi-induced TLR2 transcription. A, SB431542 (1 μM), a specific inhibitor for TβRI, and overexpression of a dominant negative mutant of TβRII attenuated NTHi-induced TLR2 expression at the mRNA level, whereas overexpression of a wild-type TβRII enhanced NTHi-induced TLR2 expression at the mRNA level in HeLa cells, as assessed by performing real time quantitative PCR analysis. B, overexpression of a dominant negative mutant of TβRII inhibited NTHi-induced TLR2 expression at the mRNA level, whereas overexpression of a wild-type TβRII enhanced NTHi-induced TLR2 expression at the mRNA level in HeLa cells. C, TβRII knockdown using TβRII-siRNA inhibited NTHi-induced TLR2 expression at the mRNA level in HeLa cells. D, TβRII knockdown using TβRII-siRNA also inhibited NTHi-induced TLR2 expression at mRNA level in HeLa cells. E, TGF-β1-induced SBE-luc activity (left) and NTHi-induced TLR2-luc activity (right) in wild-type Mv1Lu cells expressing TβRI and TβRII but not in mutant R1B and DR26 cells lacking functional TβRI and TβRII, respectively. A TLR2-luc or SBE-luc reporter vector was transfected into Mv1Lu, R1B, or DR26 cells. NTHi or recombinant human TGF-β1 was then added to the transfected cells 48 h after transfection. After 5 h, the cells were harvested for luciferase assay. F, overexpression of a dominant negative mutant of TβRI or TβRII inhibited TGF-β1-induced SBE-luc activity (left, top panel) and NTHi-induced TLR2-luc activity (left, lower panel) in wild-type Mv1Lu cells, whereas overexpression of a wild-type TβRI or TβRII in R1B cells or TβRII in DR26 cells enhanced TGF-β1-induced SBE-luc activity (right, top panels) and NTHi-induced TLR2-luc activity (right, bottom panels). Values are the mean ± S.D.; n = 3.

TLR2 induction, we took advantage of the available R1B and DR26 cells, two cell lines that are derived from wild-type Mv1Lu cells and lack functional TβRI and TβRII, respectively (38). We first assessed the effect of TGF-β1 on SBE-dependent promoter activity in Mv1Lu, R1B, and DR26 cells. As expected, almost no TGF-β1-induced promoter activity was observed in both R1B and DR26 cells, whereas wild-type Mv1Lu cells exhibited induction of SBE-dependent promoter activity by TGF-β1 (Fig. 1E, left). We next examined the effects of NTHi on TLR2 promoter activity in the same types of cells. As shown in Fig. 1E (right), NTHi-induced TLR2 promoter activity was abolished in both R1B and DR26 cells, whereas wild-type Mv1Lu cells still exhibited induction of NTHi-induced TLR2 promoter activity. Furthermore, overexpression of a dominant negative mutant of either TβRI or TβRII inhibited TGF-β1-induced SBE activity (Fig. 1F, top) and NTHi-induced TLR2 transcription in wild-type Mv1Lu cells (Fig. 1F, bottom), respectively, whereas overexpressing wild-type TβRI and TβRII rescued their responsiveness to either TGF-β1 (Fig. 1F, top) or NTHi (Fig. 1F, bottom) in R1B and DR26 cells, respectively. Taken together, our results suggest the requirement of TGF-β receptor type I/II signaling in NTHi-induced TLR2 transcription.

TGF-β Receptor-mediated Signaling Pathway Acts as a Positive Regulator for NTHi-induced TLR2 Transcription via a Smad3/4-dependent Mechanism—Because of the importance of Smads in transducing TGF-β receptor-mediated signals into the nucleus, we next sought to determine the involvement of Smad3, one of the key receptor-activated Smads, and Smad4, the Co-Smad (common partner Smad), in NTHi-induced TLR2 transcription. As shown in Fig. 2A, overexpression of a dominant negative mutant of either Smad3 or Smad4 inhibited NTHi-induced TLR2 transcription, whereas overexpression of a wild-type Smad3 or Smad4 enhanced it, thereby suggesting the involvement of Smad3/4 in
Collectively, these results demonstrated that the TGF-β signal transduction between phosphorylated Smad3 and Smad4 and nuclear translocation of TLR2 transcription. Because complex formation induced TLR2 expression at mRNA level in HeLa cells, as assessed by performing real time quantitative PCR analysis. E, overexpression of a wild-type Smad4 enhanced NTHi-induced TLR2 expression at mRNA level in Smad4-deficient MDA-MB468 cells. Values are the mean ± S.D. (n = 3).

NTHi-induced TLR2 transcription. To further confirm the involvement of Smads in TLR2 transcription, we examined TLR2 induction by NTHi in MDA-MB468 (hereafter MB468), a Smad4-deficient breast cancer epithelial cell line (39) (Fig. 2B, top). As expected, overexpression of wild-type Smad4 enhanced NTHi-induced TLR2 transcription (Fig. 2B, bottom). Collectively, these results demonstrated that the TGF-β receptor-mediated signaling pathway acts as a positive regulator for NTHi-induced TLR2 transcription via a Smad3/4-dependent mechanism.

NTHi Activates TGF-β-Smad Signaling Probably via an Autocrine-independent Mechanism—Although we have demonstrated that TGF-β signaling acts as a positive regulator for NTHi-induced TLR2 transcription, it is still unclear how TβRII/II-Smad3/4 signaling is activated by NTHi in regulation of TLR2 transcription. Because complex formation between phosphorylated Smad3 and Smad4 and nuclear translocation of Smad3/4 are known as important steps in TGF-β-mediated transcriptional activity (1–7), we first sought to confirm whether NTHi indeed activates TβRII/II-Smad3/4 signaling like TGF-β does by evaluating the effect of NTHi on phosphorylation of Smad3 and nuclear translocation of Smad3/4. As shown in Fig. 3A, NTHi, like TGF-β1, induced rapid phosphorylation of Smad3 and also induced nuclear translocation of phosphorylated Smad3, Smad3, and Smad4. Notably, the induced phosphorylation of Smad3 occurred at as early as 1–2 min. To confirm whether NTHi also induces Smad3/4-dependent transcriptional activity, we assessed the effect of NTHi on SBE-dependent promoter activity using the SBE luciferase reporter and TGF-β-responsive promoter activity of a PAI-1-Luc in HeLa cells. When we exposed the transfected cells to NTHi or TGF-β1, SBE-driven luciferase activity greatly increased in cells treated with NTHi or TGF-β1 (Fig. 3B), confirming that NTHi indeed activates the TGF-β-Smad3/4 signaling pathway. To further determine whether TGF-β-Smad3/4 signaling is activated directly by an NTHi-derived TGF-β mimicry molecule or indirectly by NTHi-induced TGF-β autocrine signaling, we next assessed whether NTHi actually induces the transcription of three major TGF-β family members, TGF-β1, -β2, and -β3. As shown in Fig. 3C, NTHi did not up-regulate TGF-β1, -β2, and -β3 transcription at the mRNA levels in HeLa cells. Consistent with this finding, NTHi did not induce any detectable increase in TGF-β1, -β2, and -β3 in the conditioned medium of the epithelial cells as assessed by performing ELISA analysis (Fig. 3D). Taken together, it is likely that NTHi may directly activate TβRII/II-Smad3/4 signaling via a TGF-β autocrine-independent mechanism. To further determine whether the NTHi-derived TGF-β-mimicry molecule is responsible for the activation of TGF-β-Smad3/4 signaling, we assessed the effect of NTHi lysates pretreated with TGF-β-neutralizing antibody or control antibody on TLR2 transcription. As shown in Fig. 3E, TGF-β1-induced PAI-1 transcription was attenuated by pretreatment of TGF-β1 with TGF-β-neutralizing antibody, thus validating the efficiency of the TGF-β neutralizing antibody in blocking TGF-β signaling. Moreover, pretreatment of NTHi lysates with the same TGF-β-neutralizing antibody reduced its ability to induce TLR2 transcription, thereby suggesting that direct activation of TβR-mediated signaling by NTHi lysates is involved in TLR2 up-regulation. To further confirm whether the NTHi-derived TGF-β-mimicry molecule is indeed involved in activating TβRII/II-Smad3/4 signaling, we next assessed the effect on TLR2 transcription of NTHi lysates. As shown in Fig. 3F, NTHi did not up-regulate TLR2 transcription in cells treated with immunodepleted TGF-β1 using TGF-β antibody (Fig. 3F, left). Likewise, immunodepletion of NTHi-derived TGF-β mimicry molecule using TGF-β antibody reduced NTHi-induced TLR2 promoter activity (Fig. 3F, right). Together, these data suggest that TβR-mediated signaling is probably activated by an NTHi-derived TGF-β mimicry molecule via an autocrine-independent mechanism.

Functional Cooperation of Smad3/4 with NF-κB Appears to Mediate NF-κB-dependent TLR2 Transcription Induced by NTHi—Because activation of NF-κB is required for NTHi-induced TLR2 transcription and functional cooperation between Smad3/4 and other transcription factors, such as NF-κB, plays an important role in mediating transcription of a variety of
genes, we sought to determine whether the TGF-β-Smad3/4 signaling pathway mediates TLR2 transcription via cross-talk with NF-κB. We first confirmed the requirement of NF-κB pathway in TLR2 induction by NTHi. As shown in Fig. 4A, overexpression of a transdominant mutant form of IκBα or a dominant negative mutant form of IKKβ inhibited activation of NF-κB and TLR2 induction by NTHi. To determine whether the TGF-β-Smad3/4 signaling pathway is involved in NF-κB activation, we first evaluated the effects of overexpressing dominant negative mutants of TβRII, Smad3, and Smad4 on NTHi-induced NF-κB-driven promoter activity. As shown in Fig. 4B, overexpression of dominant negative mutants of TβRII, Smad3, and Smad4 inhibited NTHi-induced NF-κB activation. To further confirm the involvement of TGF-β-Smad3/4 signaling pathway in NF-κB activation, we examined NTHi-induced NF-κB activation in Smad4-deficient MB468 cells. As expected, overexpression of wild-type Smad4 greatly enhanced NTHi-induced NF-κB promoter activity (Fig. 4C). Moreover, activation of TGF-β-Smad3/4 signaling by TGF-β1 or co-expression with wild-type Smad3 and Smad4 greatly enhanced NTHi-induced NF-κB activation (Fig. 4D). Taken together, the TGF-β-Smad3/4 signaling pathway is directly involved in NTHi-induced NF-κB activation. Next, we investigated whether the Smad3/4 pathway may be positively involved in TLR2 transcription. These data indicate that the TGF-β-Smad3/4 signaling pathway mediates TLR2 transcription probably via functional cooperation with NF-κB.

**FIGURE 3.** NTHi activates TβR-Smad signaling probably via an autocrine-independent mechanism. A, treatment with NTHi or TGF-β1 (1 ng/ml) induced phosphorylation of Smad3 in HeLa cells, as assessed by Western blotting using anti-phosphorylated Smad3 antibody (top panels). Moreover, NTHi and TGF-β1 also induced nuclear translocation of phospho-Smad3, Smad3, and Smad4 (bottom panels). Representative fields of phospho-Smad3, Smad3, and Smad4 fluorescence (bottom panels) were shown in HeLa cells that were treated with NTHi or TGF-β1 (1 ng/ml) for 60 min, respectively. B, NTHi and TGF-β1 (1 ng/ml) induced Smad-regulated promoter activity of SBE-luc and TGF-β-responsive promoter activity of PAI-1-luc in HeLa cells. C, NTHi did not induce TGF-β1, -β2, or -β3 expression at mRNA levels in HeLa cells. D, NTHi did not induce any detectable increase in TGF-β1, -β2, and -β3 in the conditioned medium of HeLa cells as assessed by TGF-β ELISA assay. E, pretreatment of NTHi or TGF-β1 (1 ng/ml) with the TGF-β-neutralizing antibody (1 μg/ml) reduced its ability to induce TLR2 or PAI-1 expression in HeLa cells, respectively. F, up-regulation of either PAI-1 or TLR2 promoter activity was reduced in HeLa cells treated with either the TGF-β1 or NTHi lysates, in which the TGF-β1 or the TGF-β-like factors were already immunodepleted using anti-TGF-β antibody. Values are the mean ± S.D. (n = 3).
TLR2 induction via negative cross-talk with the p38 MAPK pathway, we next evaluated the effects of inhibiting the TβR-Smad3/4 pathway on NTHi-induced activation of p38 and its upstream kinases MKK3/6 by performing Western blot analysis using antibodies against phosphorylated p38 and MKK3/6. NTHi-induced activation of p38, but not MKK3/6, was greatly enhanced by perturbing TβR-Smad3/4 signaling using multiple approaches, including using TβRI inhibitor, SB431542 (Fig. 5B), overexpressing the dominant negative mutant form of either TβRI or TβRII (Fig. 5C and D), knockdown of either TβRI or TβRII expression using the siRNA approach (Fig. 5E), and overexpressing the dominant negative mutant form of either Smad3 or Smad4 (Fig. 5F). In agreement with these results, the addition of exogenous TGF-β1 in parallel with dual specificity phosphatases collectively termed MAPK phosphatases, represents a key protein phosphatase that dephosphorylates and inactivates p38 MAPK (14, 40), we first determined whether treatment of epithelial cells with either TGF-β1 or NTHi induces MKP-1 expression. As shown in Fig. 6B, TGF-β1 or NTHi indeed induced MKP-1 expression at the protein level (Fig. 6B). To determine whether MKP-1 induction by NTHi is mediated via TβR-Smad3/4 signaling, we next evaluated the effects of inhibiting TβR-Smad3/4 signaling on NTHi-induced MKP-1 expression by performing Western blot analysis. As shown in Fig. 6C, MKP-1 induction by NTHi was inhibited by perturbing TβR-Smad3/4 signaling using various approaches, including using the TβRI inhibitor SB431542 and overexpressed NTHi attenuated NTHi-induced phosphorylation of p38 but not MKK3/6 (Fig. 5G). In contrast to the role of TGF-β-Smad signaling in negatively regulating p38 activation, perturbing IKKβ-IκBα signaling did not alter NTHi-induced p38 phosphorylation (Fig. 5H). Thus, these results indicate that the TβR-Smad3/4 signaling pathway acts as a positive regulator for NTHi-induced TLR2 transcription probably via negative cross-talk with p38 MAPK but not its upstream kinase MKK3/6.

TβR-Smad3/4 Signaling Pathway Is Positively Involved in NTHi-induced TLR2 Transcription via MKP-1-dependent Inhibition of p38 MAPK—One key issue that has yet to be addressed is how TβR-Smad3/4 signaling down-regulates NTHi-induced p38 activation. We first determined the possible involvement of de novo protein synthesis in NTHi-induced TLR2 transcription using a well known protein synthesis inhibitor, cycloheximide. As shown in Fig. 6A, NTHi-induced TLR2 up-regulation was inhibited in HeLa cells pretreated with cycloheximide, indicating that de novo protein synthesis is positively involved in NTHi-induced TLR2 transcription. Because of this finding and the evidence that TβR-Smad3/4 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. On the basis of the fact that MKP-1, a member of a key class of positive regulator for NTHi-induced TLR2 transcription probably via negative cross-talk with p38 MAPK but not its upstream kinase MKK3/6.

FIGURE 4. Functional cooperation of Smad3/4 with NF-κB appears to mediate NF-κB-dependent TLR2 transcription induced by NTHi. A, overexpressing a transdominant mutant form of IκBα or a dominant negative mutant form of IKKβ inhibited activation of NF-κB and TLR2 induction by NTHi. B, overexpression of dominant negative mutants of TβRII, Smad3, and Smad4 inhibited NTHi-induced NF-κB activation in Smad4-deficient MDA-MB468 cells. C, overexpression of a wild-type Smad4 enhanced NTHi-induced NF-κB activation in Smad4-deficient MDA-MB468 cells. D, activation of TGF-β signaling by TGF-β1 (1 ng/ml) or overexpression of wild-type Smad3 and Smad4 markedly enhanced NTHi-induced NF-κB activation. E, coexpression of Smad3, Smad4, p65, and p50 greatly induced NF-κB activation and TLR2 transcription. Values are the mean ± S.D. (n = 3).
pressing dominant negative mutant forms of TβRI, TβRII, Smad3, and Smad4. In contrast to the role of TGF-β-Smad signaling in regulating MKP1 expression, perturbing IKKβ signaling did not alter NTHi-induced MKP-1 transcription (Fig. 6D). Therefore, it is evident that TβR-Smad3/4 signaling is indeed involved in MKP-1 induction by NTHi, which in turn leads to down-regulation of p38 activity.

To further confirm the involvement of MKP-1 in NTHi-induced TLR2 transcription, we next assessed the effect of Ro-31-8220, a chemical inhibitor for MKP-1 transcription, on NTHi-induced TLR2 transcription. As expected, Ro-31-8220 indeed reduced NTHi-induced TLR2 up-regulation (Fig. 6E). Consistent with this result, overexpression of the antisense MKP-1 expression vector inhibited NTHi-induced TLR2 transcription, whereas overexpression of a wild-type MKP-1 expression vector inhibited NTHi-induced TLR2 transcription (Fig. 6F). To further confirm whether MKP-1 is indeed involved in TLR2 induction, we then assessed the effect of MKP-1 knockdown by siRNA approach on NTHi-induced TLR2 transcription. As shown in Fig. 6G, MKP-1 knockdown by MKP-1 siRNA indeed inhibited NTHi-induced TLR2 transcription and enhanced NTHi-induced p38 phosphorylation as expected. Consistent with the involvement of MKP-1 in TLR2 induction by NTHi at the mRNA level as assessed by real time quantitative PCR, pretreatment in HeLa cells with Ro-31-8220 also reduced TLR2 induction at the protein level (Fig. 6H). Collectively, these data suggest that TβR-Smad3/4 signaling pathway also mediates NTHi-induced TLR2 transcription via MKP-1-dependent inhibition of p38 MAPK.

**TβR-Smad3/4 Signaling Pathway Is Also Positively Involved in Mediating NTHi-induced Transcription of TLR2, TNF-α, and IL-1β as Well as Host Defense in Vivo**—To further confirm whether TβR-Smad3/4 signaling is also positively involved in TLR2 induction by NTHi in vivo, we next determined the effects of SB431542, a specific TβRI inhibitor, and Ro-31-8220, a chemical inhibitor for MKP-1 transcription, on NTHi-induced TLR2 transcription in the lungs of the mice. As shown in Fig. 7A and B, TLR2 induction by NTHi was attenuated by SB431542 and Ro-31-8220, indicating that the TβR-Smad3/4 signaling path-
Positive Regulation of TLR2 by TGF-β Signaling

AUGUST 4, 2006•VOLUME 281•NUMBER 31
JOURNAL OF BIOLOGICAL CHEMISTRY 22405

way also acts as a positive regulator for NTHi-induced TLR2 transcription in vivo.

Having demonstrated the positive involvement of TβR-Smad3/4 signaling in mediating NTHi-induced TLR2 transcription via MKP-1-dependent inhibition of p38 MAPK, the physiological relevance of the TGF-β signaling-mediated TLR2 induction by NTHi in vivo, however, still remains unclear. Because of the importance of TLR2 in mediating NTHi-induced inflammatory and immune responses, we hypothesized that activation of the TβR-Smad3/4 signaling pathway by NTHi also mediates NTHi-induced transcription of several key cytokines, such as TNF-α and IL-1β. To test this hypothesis, we assessed the effects of SB431542 on NTHi-induced TNF-α and IL-1β transcription in the lungs of the mice. As shown in Fig. 7, C and D, NTHi-induced TNF-α and IL-1β transcription was also inhibited by SB431542, thereby demonstrating that NTHi-induced TLR2 transcription via TβR-Smad3/4 signaling does play an important role in mediating induction of cytokines by NTHi. To further determine the in vivo role of TGF-β-mediated TLR2 regulation in host defense, we next evaluated the effect of perturbing TGF-β-signaling on bacterial clearance from the lung of the wild-type and TLR2-deficient mice upon bacterial inoculation. As shown in Fig. 7E, preadministration of TGF-βR inhibitor SB431542 resulted in increased bacterial counts in the lung of WT mice by over 3-fold as compared with vehicle-treated mice, indicating that TGF-β signaling plays a critical role in bacterial clearance from the lung. Moreover, bacterial counts increased by ~2-fold in the lung of TLR2-deficient mice as compared with that in WT mice, suggesting that TLR2 plays an important role in bacterial clearance. Interestingly, preadministration of TGF-βR inhibitor SB431542 in TLR2-deficient mice only resulted in ~60% increase in bacterial accounts as compared with an ~3-fold increase in WT mice. Thus, it is clear that TGF-β-mediated TLR2 regulation is at least in part responsible for the bacterial clearance. It should be noted that TGF-β signaling also appears to be partially involved in bacterial clearance via a mechanism independently of TLR2. Given the complex role of TGF-β signaling in regulating host immune and defense response and the experimental evidence we showed in Fig. 7E, it is reasonable to conclude that TGF-β-mediated TLR2 regulation does play an important role in host defense in vivo.
DISCUSSION

In summary, we have shown that TGF-β receptor-mediated signaling pathways acts as a positive regulator for bacterium NTHi-induced TLR2 transcription via a Smad3/4-dependent mechanism (Fig. 8). We also showed that TβR-mediated signaling is probably activated by an NTHi-derived TGF-β mimicry molecule via an autocrine-independent mechanism. Moreover, our data indicate that TGF-β receptor-Smad3/4 signaling pathways are positively involved in TLR2 induction by NTHi via a dual mechanism. One mechanism involves functional cooperation between the TGF-β/Smad3/4 pathway and NF-κB pathway. Another one involves MKP-1-dependent inhibition of p38 MAPK, a negative regulator for TLR2 induction. Finally, we showed that TβR-Smad3/4 signaling pathway also acts as a positive regulator for NTHi-induced TLR2 transcription in vivo and that TGF-β-mediated TLR2 regulation does plays an important role in host defense. Our study thus provides new insights into the role of TGF-β signaling in positively regulating host defense response by controlling the expression level of TLR2 during bacterial infections and may help to further develop new therapeutic targets for the treatment of NTHi infections.

One important finding in the present study is that TGF-β-mediated signaling, generally known to play an important role in suppressing immune response (1–7), actually acts as a positive regulator for host defense and immune response by increasing the expression of host defensive receptor TLR2 in respiratory bacterial infections (19–20, 22). Our finding may have some important implications for the tight regulation of host defense and immune response in the molecular pathogenesis of bacterial infections. TLRs are well known to play important roles in host defense against invading microbial pathogens. Among all known TLRs, TLR2 has been shown to be critically involved in responding to a variety of Gram-positive bacterial products, including peptidoglycan, lipoprotein, lipoteichoic acid, and lipoarabinomannan (19–23). The importance of TLR2 in host defense was further highlighted by the studies from knock-out mice showing decreased survival of TLR2-deficient mice after infection with Gram-positive S. aureus (26).

Moreover, our recent study demonstrated that TLR2 also plays a key role in activating host immune and inflammatory response by surface lipoprotein from the Gram-negative bacterium NTHi, a major cause of otitis media, and exacerbation of chronic obstructive pulmonary diseases (27–31). Thus, it is
clear that TLR2 plays a crucial role in host defense against both Gram-positive and -negative bacteria. However, TLRs have also been implicated in some immune mediated and inflammatory diseases due to excessive immune responses. To avoid detrimental immune and inflammatory response, the host immune system needs to constantly strike a balance between activation and inhibition of immune response. One important way to achieve such a balance is to tightly control the expression level of TLRs. Given the direct evidence presented in the current study that TGF-β signaling is positively involved in TLR2 induction by bacteria and the bacterial clearance from the lung, it is likely that TGF-β signaling may also play a critical role in tightly regulating the host defense and immune response, especially during bacterial infections.

Another interesting finding in this study is the negative cross-talk between TGF-β-Smad3/4 signaling pathway and the p38 MAPK pathway. Recently, there is increasing evidence for the involvement of p38 MAPK in TGF-β signaling-mediated cellular responses. Whereas most of these studies have demonstrated an important role of TGF-β signaling in activation of p38 MAPK in a Smad-independent manner (42, 43), the negative regulation of p38 MAPK by TGF-β-Smad signaling still remains largely unexplored. In the current study, we provided first-hand evidence for the negative regulation of the bacteria-induced p38 activation by TGF-β-Smad3/4 signaling in a MKP-1-dependent manner. This finding should further enhance our understanding of the complex signaling mechanism underlying the functional interactions between TGF-β-Smad signaling pathway and the p38 MAPK pathway.

A third interesting finding in this study is that TGF-βR appears to act as a cellular receptor for the TGF-β mimicry molecule derived from bacterial pathogen NTHi. This result, although rather unexpected, may provide a novel insight into the role of TGF-βR in the molecular pathogenesis of bacterial infections. In view of the biological actions of TGF-βR, significant progress has been made toward fully understanding the role of TGF-βR in regulating 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 whether TGF-β is also involved in mediating host defense response by acting as a host receptor for bacteria-derived mimicry molecule in infectious diseases. Despite some studies showing that TGF-βR may act as a cellular receptor for parasite T. cruzi, little is known about the role of TGF-βR in acting as a receptor for bacterial pathogen (8). Thus, our studies demonstrate for the first time that TGF-β mimicry molecule derived from NTHi, a major human bacterial pathogen, may activate TGF-βR signaling that in turn leads to up-regulation of TLR2, probably via a TGF-βR autocrine-independent mechanism. It should be noted that a functional mimicry of TGF-β activity may be more likely involved in activating TGF-βR signaling, since a sequences analysis of the available NTHi genome did not show any high homology between the known NTHi genes and the TGF-β family members. In addition, our data do not rule out the possible involvement of the latent TGF-β stored in the extracellular matrix that might be activated by NTHi and then cross-talk with TβRII. Finally, it is still unclear whether other TGF-β family members are involved in mediating the negative regulation of NTHi-induced TLR2 transcription in an autocrine-dependent manner. Future studies will focus on determining the molecular identity of the NTHi-derived TGF-β mimicry molecule mimicking the host TGF-β molecule and whether NTHi also activates the latent TGF-β stored in the extracellular matrix that, in turn, leads to the activation of TGF-β signaling.

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Positive Regulation of TLR2 by TGF-β Signaling

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