Role of p38 MAP Kinase and Transforming Growth Factor-β Signaling in Transepithelial Migration of Invasive Bacterial Pathogens*

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Streptococcus pneumoniae and Haemophilus influenzae are human pathogens that often asymptomatically colonize the mucosal surface of the upper respiratory tract, but also occasionally cause invasive disease. The ability of these species to traverse the epithelium of the airway mucosa was modeled in vitro using polarized respiratory epithelial cells in culture. Migration across the epithelial barrier was preceded by loss of transepithelial resistance. Membrane products of S. pneumoniae that included lipoteichoic acid induced disruption of the epithelial barrier in a Toll-like receptor 2-dependent manner. This result correlates with a recent genetic study that associates increased TLR2 signaling with increased rates of invasive pneumococcal disease in humans. Loss of transepithelial resistance by the TLR2 ligand correlated with activation of p38 MAPK and TGF-β signaling. Activation of p38 MAPK and TGF-β signaling in epithelial cells upon nasal infection with S. pneumoniae was also demonstrated in vivo. Inhibition of either p38 MAPK or TGF-β signaling was sufficient to inhibit the migration of S. pneumoniae or H. influenzae. Our data shows that diverse bacteria utilize common mechanisms, including MAPK and TGF-β signaling pathways to disrupt epithelial barriers and promote invasion.

Epithelial surfaces form tight barriers that are highly effective at blocking penetration by most microbes. Mucosal surfaces in particular are constantly exposed to a variety of microbial species, yet few of these species are proficient in causing invasive infection from this niche. Invasion from the mucosa requires attachment to the epithelial surface, transepithelial migration, and evasion of immune mechanisms that target penetrating organisms. In the case of bacterial pathogens of the human respiratory tract, two of the members of the microbial flora commonly associated with invasive infection are the Gram-positive species Streptococcus pneumoniae, and the Gram-negative species Haemophilus influenzae. Although distantly related, both share the ability to colonize the mucosal surface of the upper airway and directly invade host barriers at this site. For S. pneumoniae this event is particularly common during infancy and is referred to as occult bacteremia. When isolates of either of these species express a capsular polysaccharide, which inhibits clearance by complement, antibody and phagocytes, they are capable of surviving in the bloodstream and causing life-threatening diseases including septicemia and meningitis. Encapsulated S. pneumoniae and H. influenzae remain among the leading causes of infectious diseases with an estimated annual worldwide mortality in children of 1.0 and 0.4 million, respectively (www.who.int).

Host and bacterial processes that allow for adherence to epithelial surfaces and survival in the bloodstream have been studied in detail. In contrast, it is not understood how these pathogens pass through the epithelial cell barrier. Many invasive pathogens are thought to transit from the apical to basolateral surface through an intracellular route. The expression of a thick capsular polysaccharide layer, however, inhibits invasion into host cells (1, 2). In contrast, it has been suggested that expression of tissue-damaging toxins, such as the pore-forming, cholesterol binding cytotoxin, pneumolysin, expressed by S. pneumoniae, facilitates passage through tissue barriers (3).

Another possibility is that the inflammatory responses to these organisms contribute to the breakdown of epithelial defenses and its barrier function. Innate immune response of human epithelial cells in culture to S. pneumoniae and H. influenzae has been examined. H. influenzae has been reported to induce responses through pattern recognition receptors, Toll-like receptors (TLR)2 and 4, which bind its lipoproteins and lipopolysaccharide, respectively (4–6). S. pneumoniae also interacts with TLR2 via its lipid anchored membrane components (7, 8) and TLR4 via pneumolysin (9). Recognition of these bacterial products by TLRs leads to signaling events that result in activation of NFκB and p38 MAP kinase (p38 MAPK) with subsequent proinflammatory responses (4, 6, 10, 11). Mice lacking TLR2, for example, are more susceptible to overwhelming infection with S. pneumoniae (12). In addition, both species express phosphorylcholine on their surface and can bind and

2 The abbreviations used are: TLR, Toll-like receptor; MAPK, mitogen-activated protein kinase; LTA, lipoteichoic acid; TER, transepithelial resistance; cfu, colony forming unit; PBS, phosphate-buffered saline; IL, interleukin; siRNA, small interfering RNA; TGF, transforming growth factor.

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conducting signaling through mimicry of platelet activating factor and ligation of its receptor (2, 13).

Despite the overall protective function of the innate immune response to pathogens it is conceivable that certain pathogens take advantage of the immune response. For instance, a recent clinical study of >6000 individuals with invasive infection, including patients with *S. pneumoniae*, examined the effect of polymorphisms in the adaptor protein Mal (also known as TIRAP), which mediates downstream signaling of TLRs including TLR2 (14). This study showed that the Mal S180L variant attenuated TLR2 signal transduction and was associated with a lower rather than higher incidence of pneumococcal and other invasive infections (14).

The purpose of this study was to characterize bacterial and host factors that allow bacteria to cross an epithelial barrier. These events were modeled using polarized human respiratory tract epithelial cells in culture. We demonstrate that bacterial recognition by TLR2 leads to p38 MAPK and transforming growth factor (TGF)-β signaling that induces a disruption of the barrier function and allows for transepithelial migration.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Products**—An encapsulated strain of *S. pneumoniae* D39, as well as its unencapsulated and unencapsulated pneumolysin-deficient derivatives, were grown as described (16). A clinical isolate of nontypeable *H. influenzae*, H233, was grown in brain heart infusion media supplemented with 2% Fildes enrichment and 2 μg/ml NAD (sBHI; Difco Laboratories). Phosphorylcholine expressing and non-expressing variants were previously described (17). *S. pneumoniae* lipoteichoic acid (LTA) was obtained from W. Fischer and isolated as described (18). *Staphylococcus aureus* LTA, Pam3CSK4, and Pam2CSK4 were purchased from InvivoGen.

**Cell Lines and Culture Conditions**—16HBE14o− (19), HEK293 and Caco-2 cells were grown in Dulbecco’s modified Eagle’s media (Invitrogen), 10% fetal bovine serum (HyClone), 2 mm l-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin. Polarized epithelial cell layers were grown as liquid–liquid interface cultures. 16HBE and Caco-2 cells were plated in 6.5-mm, 0.4-μm pore size transwell inserts for stimulation assays or in 24-mm, 3-μm pore size inserts for migration assays (Costa). The cells were cultured for at least 5 days and used for experiments when confluent and a transepithelial resistance (TER) of at least 300 Ohm cm² was reached. TER measurements were performed using an ohmmeter (EVOM; World Precision Instruments). Lactic dehydrogenase release was measured using a cytotoxicity detection kit (Roche).

**Migration Assay**—*S. pneumoniae* was grown to mid-log phase (A₆₂₀ = 0.2) and diluted in tissue culture media (Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum) without antibiotics. At time 0, the media on the apical side of the transwell inserts (24-mm, 3-μm pore size) was replaced with bacterial suspension (2 × 10⁴ colony forming units (cfu)/ml), and replaced with culture media after 10 and 14 h. During this period the bacterial density in the apical compartment expanded by ~100-fold. Every 2 h, 100 μl of media was removed from the basolateral compartment and plated for quantitative culture and 100 μl of fresh media was replaced in the basolateral compartment. A transwell was considered to be passed by bacteria at the initial time point that one or more organisms could be detected in the basolateral media. To investigate the effect of SB203580 (BIOSOURCE) and SB431542 (BIOSOURCE) on migration time, transwells were preincubated with 10 μM of the inhibitors for 1 h before inoculation of bacteria (4 × 10⁶ cfu/ml *S. pneumoniae*, 10⁶ cfu/ml *H. influenzae*). To confirm that differences in migration time were not due to differential bacterial growth, aliquots of the apical compartment were cultured at different time points and plated at appropriate dilutions.

**Fractionation of Bacteria**—Unencapsulated *S. pneumoniae* (D39) were grown as a 400-ml culture to an A₆₂₀ of 0.4, harvested by centrifugation, washed, and resuspended in 20 ml of phosphate-buffered saline (PBS). Bacteria were lysed using a French press. The lystate was centrifuged at 100,000 × g for 60 min. The pellet containing the cell wall and membrane fraction (referred as membrane fraction) was resuspended in 10 ml of PBS, diluted 1/40 in culture media, and applied to 16HBE on transwells. For further studies, the membrane fraction was treated for 60 min, with or without 400 μg/ml Proteinase K or for 12 h at 4 °C, or with or without 0.05 M periodate (Sigma) in sodium acetate buffer, 50 mm, pH 4.2. The fractions were washed twice with PBS and resuspended in PBS before addition to cells. Efficient digestion of proteins was confirmed by SDS-PAGE and silver staining. Where indicated, the membrane fraction was treated for 120 min with or without 0.2 μM NaOH at 37 °C and neutralized with HCl.

**Stimulation of Cells with Bacteria and Bacterial Factors**—16HBE and HEK293 cells cultured as submersed cultures or on transwells as described above were incubated with *S. pneumoniae*, *H. influenzae*, LTA from *S. aureus*, Pam3CSK4, Pam2CSK4, the membrane fractions of *S. pneumoniae*, *S. pneumoniae* LTA, and TGF-β1 (BIOSOURCE) at the indicated densities and concentrations. TER was measured at the indicated time points and supernatants were collected to measure interleukin (IL) 8 release. To investigate the effect of the p38 MAPK inhibitors SB203580 (BIOSOURCE) and SB202190 (BIOSOURCE) and the TGF-β1 inhibitor SB431542 (BIOSOURCE) on TER, the cells were preincubated for 30 min with the inhibitor (each at a final concentration of 10 μM) before different stimuli were applied.

**Immunofluorescence Microscopy**—16HBE cells plated on collagen-coated chamber slides or cultured on transwells were washed with PBS, fixed in 5% paraformaldehyde in PBS for 10 min at room temperature, washed again, and permeabilized with 1% Triton X-100 for 10 min. Cells were incubated with primary antibodies against E-cadherin (Zymed Laboratories) or with an antiserum against whole *S. pneumoniae* or p-Smad2/3 (Santa Cruz Biotechnology) for 1 h at room temperature. Cells were then washed, incubated with fluorescein-conjugated secondary antibodies (Jackson ImmunoResearch) for 2 h at room temperature, washed again, and mounted with mounting media. Image analysis was carried out using an Olympus fluorescence microscope or a confocal laser-scanning microscope.

**Mouse Model of Nasopharyngeal Colonization and Immunohistochemistry**—Six- to eight-week-old female C57BL/6j mice were obtained from Taconic and housed in accordance
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with Institutional Animal Care and Use Committee protocols. The mouse model of nasopharyngeal colonization with S. pneumoniae was previously described (20). Briefly, mice were inoculated intranasally with \(1 \times 10^6\) cfu of PBS-washed, mid-log phase S. pneumoniae in 10 \(\mu\)l of PBS applied equally to both nares; mock-colonized animals were inoculated with an equivalent volume of PBS suspension. At the times indicated post-inoculation, the animals were sacrificed.

Animal tissues were obtained after decapitation, and fixed and decalcified by serial overnight incubations in 4% paraformaldehyde in PBS and Decal decalcification agent (Decal Corporation). Tissue to be embedded in paraffin was dehydrated with a series of ethanol and xylene, embedded in paraffin, and 7-\(\mu\)m sections were cut. Sections were rehydrated through xylene and ethanol and stained with hematoxylin. For immunohistochemical studies, slides were microwaved in 10 mM citric acid buffer, pH 6.0, and then endogenous peroxidases were quenched with 3% hydrogen peroxide. Endogenous biotin was blocked with an Avidin-Biotin blocking kit (Vector Laboratories) followed by peptide blocking with protein blocking reagent (Coulter/Immunotech, Miami, FL). \(p\)-p38 MAPK (Cell Signaling) and \(p\)-Smad2/3 (Santa Cruz Biotechnology) were detected with primary antibodies diluted 1:200 in PBS plus 0.1% bovine serum albumin, 0.2% Triton X-100. A biotinylated anti-rabbit secondary or anti-goat secondary (Vector Laboratories) was added followed by avidin-horseradish peroxidase ABC reagent (Vector Laboratories). Signal was developed using the DAB kit (Vector Laboratories). Imaging was performed on a Nikon E600 Eclipse microscope equipped with a high-resolution CCD digital camera (CoolSnap CF, Roper Scientific) with Nomarski optics.

Western Blotting—16HBE cells were lysed in SDS sample buffer and aliquots with equivalent total protein concentrations were separated on a 10% polyacrylamide gel. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore) and probed for phosphorylated p38 MAPK using the PhosphoPlus p38 MAPK antibody kit (Cell Signaling). Afterward, blots were stripped and reprobed for total p38 MAPK.

Enzyme-linked Immunosorbent Assay—Concentrations of IL-8 and TGF-\(\beta\)1 were determined using a commercially available enzyme-linked immunosorbent assay (Pharmingen OptEIA). Conditions were compared by one-way analysis of variance with Tukey test for multiple comparisons (Prism GraphPad software).

RNA-mediated Interference—Small interfering RNA oligonucleotides against TLR2 (ID111285) and a control siRNA with no similarity to any human gene sequence were designed and synthesized by Ambion. Transfection of Caco-2 cells with siRNA was performed using the Nucleofector System (Amaxa) according to the manufacturer’s protocol. Following transfection, cells were plated at a density of \(5 \times 10^5\) cells/well on collagen-coated transwells (6.5-mm, 0.4-\(\mu\)m pore size). 48 h after transfection, cells were stimulated with Pam2CSK4 or S. aureus LTA. TER was measured at the indicated time points. 72 h after transfection, cells were lysed in SDS sample buffer and subjected to Western blot analysis as described using a monoclonal antibody to human TLR2 (InvivoGen) and glyceraldehyde-3-phosphate dehydrogenase (Abcam).

Statistical Analysis—Comparisons between experimental groups were performed using Student’s \(t\) test or the analysis of variance as appropriate. Differences in the migration time were compared using a log rank test. Results were considered statistically significant for \(p < 0.05\).

RESULTS

S. pneumoniae Migrates between Polarized Respiratory Epithelial Cells—To investigate mechanisms involved in the migration of bacteria through polarized respiratory epithelial cells, we used an in vitro model consisting of the bronchial epithelial cell line 16HBE maintained as liquid-liquid interface cultures. 16HBE cells formed a polarized cell layer with a TER of 300–400 Ohm cm\(^2\).

S. pneumoniae isolate D39 and genetically defined unencapsulated mutants of this strain expressing or not expressing the cytotoxic pneumolysin were used to examine bacterial passage through the cell barrier. Fig. 1A shows the earliest time point when bacteria were detected in the basolateral compartment after addition of S. pneumoniae to the apical compartment of polarized 16HBE. All strains tested were eventually able to gain access to the basolateral compartment. However, the average migration time was higher for bacteria with capsule compared with bacteria without capsule, whereas the lack of pneumolysin did not significantly influence the migration time. Differences in migration time were not due to different bacterial growth rates as the numbers of bacteria were similar among the different strains in the apical compartments at all time points (data not shown). To investigate the effect of S. pneumoniae on barrier function, TER of polarized 16HBE was measured 8 and 20 h...
To further analyze the bacterial factors involved, the membrane fraction was digested with proteinase K. Fig. 3 shows that proteinase K digestion of the membrane fraction had minimal impact on the TER reducing activity, showing that factors other than proteins contributed to the loss of TER. The efficiency of proteinase K treatment was shown by silver staining of a polyacrylamide gel (data not shown). To examine if factors containing carbohydrates were involved in the loss of TER, the membrane fraction was treated with periodate. Periodate treatment resulted in a complete loss of activity of the membrane fraction (Fig. 3A). A prominent non-proteinaceous membrane-associated structure of Gram-positive bacteria that contains carbohydrate is LTA. LTA also contains acyl chains and can be deacylated by mild NaOH treatment. Mild NaOH treatment resulted in a complete loss of activity of the membrane fraction. Together these findings indicated that LTA was necessary to cause the loss of TER by the S. pneumoniae membrane fraction. Next, we investigated if LTA was sufficient to induce loss of TER in our polarized epithelial cell model. Polarized 16HBE were incubated with highly purified LTA from S. pneumoniae or commercially obtained LTA from S. aureus. Both LTA preparations induced a loss of TER and were as potent as the synthetic TLR2 ligand Pam3CSK (Fig. 4A).

Disruption of Polarized Layers Is Mediated by TLR2—To investigate if the membrane fraction potentially activates host cells by engaging TLR2, HEK293 cells overexpressing TLR2 were stimulated with the membrane fractions. Stimulation with the membrane fraction or a synthetic ligand of TLR2 resulted in a release of IL-8 by TLR2-expressing HEK293 cells (Fig. 3B). Furthermore, periodate and NaOH treatment of the membrane fraction resulted in a loss of activity (Fig. 3B). HEK293 cells carrying a control plasmid did not release any IL-8 after stimulation with the membrane fraction or synthetic ligand (data not shown). The preparation of purified S. pneumoniae LTA was also sufficient to induce IL-8 secretion by HEK293 cells overexpressing TLR2 but had no effect on control cells (Fig. 4B).

To further determine whether activation of TLR2 induces loss of TER, Caco-2 epithelial cells were transfected with siRNA against TLR2 or a control siRNA. siRNA studies were performed with the Caco-2 cell line because differentiated 16HBE cells were resistant to transfection with siRNA. Stimulation of Caco-2 cells with the synthetic TLR2 ligand Pam3CSK4 and LTA from S. aureus resulted in loss of TER (Fig. 5, A and B). Transfection of Caco-2 cells with siRNA directed against TLR2 blunted the reduction of TER in response to both TLR2 ligands compared with cells transfected with control siRNA. Reduced expression of TLR2 after siRNA transfection was confirmed by Western blotting (Fig. 5C).

![Image](https://example.com/image.png)

**FIGURE 2.** A. S. pneumoniae co-localize with intercellular junctions. 12 h after inoculation of differentiated 16HBE cells with bacteria the tissue was fixed and co-localization of S. pneumoniae (red) with E-cadherin (green) was shown by confocal microscopy (arrows). Magnification ×600. B, inhibition of p38 MAPK with SB203580 (10 μM) results in maintenance of the actin structure in 16HBE after inoculation with S. pneumoniae membrane fraction (m.f.). Controls are sham-infected cells.
p38 MAPK and TGF-β Pathways Are Activated by Bacteria—Next, we addressed whether the effects on epithelial cells by S. pneumoniae and LTA correlate with activation and phosphorylation of p38 MAPK. Fig. 6A shows that S. pneumoniae and S. pneumoniae LTA were sufficient to increase phosphorylation of p38 MAPK in differentiated 16HBE cells. To examine if p38 MAPK was involved in the loss of TER induced by bacteria and LTA, polarized cells were preincubated with the p38 MAPK inhibitor SB203580. Inhibition of p38 MAPK completely blocked the loss of TER induced by S. pneumoniae, S. pneumoniae LTA, S. aureus LTA, and the membrane fraction of S. pneumoniae (Fig. 6B). Similar results were obtained using a different p38 MAPK inhibitor, SB202190 (data not shown). Furthermore, Fig. 2B shows that inhibition of p38 MAPK resulted in maintenance of the actin structure that was disturbed by the S. pneumoniae membrane fraction, indicating that rearrangement of the cytoskeleton might contribute to the loss of barrier of the epithelium.

Because H. influenzae is also reported to stimulate TLR2-dependent phosphorylation of p38 MAPK in epithelial cells (4), the effect of isolate H233 on the TER of 16HBE cells was tested. Similar to S. pneumoniae, H. influenzae caused phosphorylation of p38 MAPK (Fig. 6A) and p38 MAPK-dependent loss of TER (Fig. 6B). The use of H. influenzae allowed us to examine the role of bacterial phosphorylcholine by employing defined strains that vary only in the expression of this surface structure. Whereas phosphorylcholine expressing variants were more adherent to the epithelial surface, transepithelial migration was minimally affected (data not shown). Results with H. influenzae demonstrated that p38 MAPK effects on epithelial integrity were not unique to S. pneumoniae. To determine whether S. pneumoniae induces p38 MAPK phosphorylation in vivo immunohistochemical staining of paraffin-embedded tissue sections from infected and mock-infected animals was performed. Fig. 6C shows that pneumococcal infection of the upper respiratory tract results in phosphorylation and nuclear translocation of p38 MAPK along the epithelial surface.

TGF-β pathways are involved in various cellular processes. For example, TGF-β induces epithelial-mesenchymal transition including loss of apical-basal polarity (21). Furthermore, the TGF-β-Smad2/3 signaling pathway acts as positive regulator for TLR2 induction by H. influenzae (22). We sought to determine whether TGF-β signaling pathways also play a role in the breakdown of barrier function induced by bacteria. First, we examined if S. pneumoniae induces phosphorylation and nuclear translocation of Smad2/3. S. pneumoniae stimulation of 16HBE cells grown on chamber slides resulted in accumulation of p-Smad2/3 in nuclei as shown by immunofluorescence microscopy (Fig. 7A). Next, we determined if TGF-β signaling was involved in the observed loss of TER. Inhibition of TGF-β signaling with the TGF-β receptor inhibitor SB431542 partially blocked the loss of TER induced by the S. pneumoniae membrane fraction, S. aureus LTA, TGF-β1, and H. influenzae (Fig. 7B). Although addition of TGF-β1 was sufficient to cause a significant loss of resistance, we were unable to detect production of TGF-β1 following bacterial infection of 16HBE cells (data not shown). This suggests that either another ligand for the TGF-β receptor was induced or that signaling by this pathway occurs downstream of ligand binding of this receptor. No effect of SB431542 on the phosphorylation of p38 MAPK was observed confirming that this inhibitor acts on a separate path-
way or downstream of p38 MAPK. Likewise, inhibition of p38 MAPK was not sufficient to block TGF-β1-induced loss of TER providing further evidence of signaling involving separate pathways (data not shown). To determine whether S. pneumoniae induces activation of TGF-β signaling pathways, in vivo immunohistochemical staining of paraffin-embedded tissue sections from infected and mock-infected animals was performed. Fig. 7C shows that infection of the upper respiratory tract results in phosphorylation and nuclear translocation of Smad2/3 along the epithelial surface.

**Passage of Bacteria through an Epithelial Barrier Is Inhibited by p38 MAPK and TGF-β Signaling Pathway Inhibitors**—As shown above, blocking of p38 MAPK and TGF-β signaling results in maintenance of the epithelial barrier function in response to bacteria and bacterial products. Next, we examined if activation of these two pathways by bacteria also contributes to the migration of bacteria through the epithelial barrier. Incubation of polarized 16HBE cells with the inhibitors SB203580 (p38 MAPK) or SB431542 (TGF-β signaling) resulted in a significantly delayed migration of S. pneumoniae and H. influenzae through the polarized 16HBE cells (Fig. 8). Differences in migration time were not due to different growth rates in the apical compartments as the bacterial density was not influenced by the inhibitors (data not shown).

**DISCUSSION**

This study analyzed the complex interrelations between host cells and the Gram-positive species S. pneumoniae or the Gram-negative species H. influenzae that allow for penetration of the airway epithelium. The main finding is that the inflammatory responses of differentiated airway epithelial cells to S. pneumoniae and H. influenzae allow bacteria to overcome their barrier function. It is shown that bacterial recognition by TLR2 leads to activation of p38 MAPK and TGF-β signaling that induces a disruption of the integrity of the barrier required for transepithelial migration. Our finding, therefore, describes a mechanism that could account for the increased incidence of invasive infection associated with genetic polymorphisms conferring increased TLR2 signaling (14).

The epithelium of the respiratory tract constitutes a physical barrier for pathogens. The tightness of this cell barrier is determined by the formation and composition of tight junctions (23). Bacteria that manage to cross the epithelial barrier can do so by either opening tight junctions and passing between the
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FIGURE 7. S. pneumoniae activates TGF-β signaling in polarized respiratory epithelial cells and inhibition of TGF-β signaling results in maintenance of barrier function. A, stimulation of 16HBE cells grown on chamber slides with S. pneumoniae membrane fraction (m.f.) and S. pneumoniae (Sp, 4 × 10^7 cfu/ml) for 1 h induced nuclear translocation of phospho-Smad2/3 as shown by immunofluorescence microscopy. B, the TGF-β signaling inhibitor SB431542 (10 μM) inhibited the loss of TER induced by S. pneumoniae (Sp, 4 × 10^7 cfu/ml), S. pneumoniae LTA (Sp LTA, 50 μg/ml), TGF-β1 (50 ng/ml), membrane fraction, and H. influenzae (Hi, 10^6 cfu/ml). TER was measured 24 h after inoculation. TER is shown as percentage of the initial TER (0 h). Values represent the mean of 4 determinations ± S.D. *, p < 0.02. C, Smad2/3 is phosphorylated and found primarily in the nuclei of olfactory epithelial cells in pneumococcal-infected (left) but not in mock infected (right) mice. Animals were sacrificed 3 h after inoculation and sections of the nasopharynx were stained with an antibody against the phosphorylated Smad2/3. Magnification ×600.

Loss of tight junctions and redistribution of occludin was also observed when intestinal epithelium was infected with Campylobacter jejuni (27). The disruption of junction structures and subsequent passage of an epithelial barrier via the paracellular route therefore seems to be a common theme among different bacteria that reside in different environments.

In our study, high densities of bacteria (>10^6 to 10^7 cfu/ml) or equivalent amounts of bacterial products were required to compromise the epithelial barrier. This correlated with the requirement for higher doses of LTA over longer periods than typically needed for stimulation of cytokine production by inflammatory cells (8, 28). Another important factor was the ability of bacteria to associate with host cells. Unencapsulated S. pneumoniae were more efficient at inducing loss of TER and passing the barrier compared with encapsulated bacteria. Bacterial capsules are known to interfere with adherence (29), and the delayed migration of encapsulated bacteria may be due to reduced bacterial-host cell interaction. Because adherence of S. pneumoniae and H. influenzae to epithelial tissue is one of the first steps in colonization and infection of the host, tight regulation of expression of the capsule might be important to colonize and disseminate in the host (1, 30). Another prominent virulence determinant expressed by S. pneumoniae is the pore-forming toxin pneumolysin. Pneumolysin is known to damage host tissue and to play a role during colonization and infection (3, 31). However, in our model, there was no significant difference in the migration time between a pneumolysin expressing S. pneumoniae strain and its pneumolysin-deficient mutant.

As bacterial adherence facilitates disruption of the epithelial barrier, it seems likely that factors on the surface of the bacteria contribute to the loss of its integrity. Taking advantage of highly purified S. pneumoniae LTA, we identified LTA as a cell surface factor that is sufficient to disrupt the epithelium. This was supported by fractionation of S. pneumoniae and the finding that non-proteinaceous membrane-associated factors containing carbohydrate and acyl chains were sufficient to disrupt the barrier. Numerous studies have shown that airway epithelium is capable of recognizing molecules with lipid anchors through the pattern recognition receptor TLR2. Furthermore, it is known that TLR2 is expressed and functional in airway epithelial cells and capable of recognizing and responding to pathogens (6, 7, 32, 33). Interestingly, Muir et al. (34) showed that TLR2 is expressed only at the apical surface of polarized epithelial cells such as 16HBE. This is consistent with our finding that cells or by migrating through the cells. To study the mechanisms involved in the passage of S. pneumoniae and H. influenzae through an epithelial barrier, we chose the bronchial epithelial cell line 16HBE grown on a collagen support to form a confluent cell layer with functional tight junctions (19). All S. pneumoniae and H. influenzae strains tested were able to disrupt the epithelial barrier as shown by a loss of TER and their ability to pass through it. As the decrease in TER preceded the passage of bacteria through the epithelium, it seems likely that the bacteria use a paracellular route to pass the epithelial barrier. This assumption was further supported by the observation that the bacteria co-localized with the cell junction factor E-catherin. Whereas Ring et al. (2) described the passage of S. pneumoniae across the blood-brain barrier via the intracellular route, our data show that this may not be the primary main route for S. pneumoniae in the respiratory tract and suggests that S. pneumoniae may use different mechanisms to pass different cell barriers. However, we cannot exclude that some bacteria might also pass the epithelial barrier via the intracellular route. Data from other groups support the idea of bacteria using the paracellular route to cross epithelial barriers. For H. influenzae, van Schilfgaarde et al. (24) showed that passage through a polarized epithelial barrier occurs between cells by paracytosis. It was also suggested that the ability of H. influenzae to penetrate the epithelial layer protects them from antibiotics and antibody-mediated bactericidal activity (25). Burkholderia cepacia also disrupts a differentiated epithelial barrier, an event associated with loss of occludin from tight junctions (26).
only apically applied bacteria caused a loss of barrier function. In addition, our results are consistent with a study showing that polarized intestinal epithelial cells respond to TLR2 ligands only when stimulated apically (35). Although LTA-TLR2 interaction was sufficient to cause the loss of TER, our study does not exclude a role for other TLRs in the response to pathogens. Given the airway epithelium recognizes S. pneumoniae via TLR2 and our result that the TLR2 ligand LTA disrupts epithelial integrity, we suggest that S. pneumoniae utilizes this pathway to penetrate epithelial defenses. H. influenzae, which is also recognized by TLR2, had a similar effect on the epithelial barrier suggesting the presence of a conserved response that may contribute to the pathogenesis of invasive infection by bacteria.

Recognition of S. pneumoniae or S. pneumoniae LTA by TLR2 leads to p38 MAPK activation that contributes to the disruption of the epithelial barrier. This is consistent with studies showing that the p38 MAPK cascade is involved in endothelial cell barrier dysfunction and actin remodeling (36, 37). In addition, we show that S. pneumoniae activated TGF-β signaling pathways, including phosphorylation and nuclear translocation of Smad proteins. Furthermore, inhibitor studies revealed that the activation of TGF-β signaling pathways also lead to a loss of TER. A variety of studies have shown that activation of the TGF-β signaling pathway results in the loss of barrier function of different tissues (38, 39). Furthermore, TGF-β mediates epithelial to mesenchymal transdifferentiation (21). Medici et al. (40) have shown that Smad-dependent and Smad-independent signaling pathways are necessary to drive epithelial-mesenchymal transition accompanied by down-regulation of tight junction factors. However, the precise mechanism by which activation of the described pathways culminated in loss of the airway epithelial barrier examined in this study remains to be revealed.

The results of this study raise the question how the TLR2 and p38 MAPK signaling cascade relates to the TGF-β pathway and how TGF-β signaling is activated by bacteria. Whereas increased levels of TGF-β1 in response to S. pneumoniae could not be demonstrated in our study, a number of related tissue factors are also ligands for its receptor. It is also possible that bacterial factors directly activate the TGF-β receptor as proposed for H. influenzae (41). However, regarding the multiplicity of factors known to activate the TGF-β signaling pathway, autocrine activation cannot be excluded. For instance, Chow et al. (42) have shown that TGF-β1 is induced in macrophages in a TLR9-dependent manner and results in an autocrine activation of TGF-β signaling. Furthermore, it is known that activation by TGF-β is cell type-specific and can result in activation of different signaling pathways including Smad- or MAPK-dependent signaling pathways (15). As inhibition of TGF-β signaling did not affect p38 MAPK phosphorylation in this study, TGF-β signaling seems to be downstream of the TLR2 and p38 MAPK signaling cascade or an independent signaling pathway. However, blocking of either p38 MAPK or the TGF-β signaling in polarized epithelial cells was sufficient to inhibit the passage of S. pneumoniae and H. influenzae through the epithelium, showing that both pathways need to be functional for the bacteria to disrupt and cross the barrier.

It remains unclear whether the loss of barrier function induced by a high density of pathogenic bacteria may also provide a protective function to the host by, for example, allowing for the ingress of antimicrobial factors, inflammatory mediators, and cells to the infected mucosal surface. Whereas avirulent species accessing the bloodstream are readily eliminated by the immune response, only certain pathogens, such as encapsulated S. pneumoniae and H. influenzae, may be able to survive this influx and persist once through the epithelial barrier. Based on our in vitro studies of transmigration through polarized bronchial epithelial cells, it appears that extracellular pathogens may take advantage of these innate defense pathways to breech tissue barriers and invade the host.

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