

Degradation Products of the Extracellular Pathogen *Streptococcus pneumoniae* Access the Cytosol via Its Pore-Forming Toxin

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ABSTRACT *Streptococcus pneumoniae* is a leading pathogen with an extracellular lifestyle; however, it is detected by cytosolic surveillance systems of macrophages. The innate immune response that follows cytosolic sensing of cell wall components results in recruitment of additional macrophages, which subsequently clear colonizing organisms from host airways. In this study, we monitored cytosolic access by following the transit of the abundant bacterial surface component capsular polysaccharide, which is linked to the cell wall. Confocal and electron microscopy visually characterized the location of cell wall components in murine macrophages outside membrane-bound organelles. Quantification of capsular polysaccharide through cellular fractionation demonstrated that cytosolic access of bacterial cell wall components is dependent on phagocytosis, bacterial sensitivity to the host's degradative enzyme lysozyme, and release of the pore-forming toxin pneumolysin. Activation of p38 mitogen-activated protein kinase (MAPK) signaling is important for limiting access to the cytosol; however, ultimately, these are catastrophic events for both the bacteria and the macrophage, which undergoes cell death. Our results show how expression of a pore-forming toxin ensures the death of phagocytes that take up the organism, although cytosolic sensing results in innate immune detection that eventually allows for successful host defense. These findings provide an example of how cytosolic access applies to an extracellular microbe and contributes to its pathogenesis.

IMPORTANCE *Streptococcus pneumoniae* (the pneumococcus) is a bacterial pathogen that is a leading cause of pneumonia. Pneumococcal disease is preceded by colonization of the nasopharynx, which lasts several weeks before being cleared by the host's immune system. Although *S. pneumoniae* is an extracellular microbe, intracellular detection of pneumococcal components is critical for bacterial clearance. In this study, we show that following bacterial uptake and degradation by phagocytes, pneumococcal products access the host cell cytosol via its pore-forming toxin. This phenomenon of cytosolic access results in phagocyte death and may serve to combat the host cells responsible for clearing the organism. Our results provide an example of how intracellular access and subsequent immune detection occurs during infection with an extracellular pathogen.

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Cytosolic detection of pathogen-associated molecular patterns is a key event in host discrimination between commensal and pathogenic microbes. While cytosolic access is critical for the pathogenesis of intracellular bacteria, access to the cytosolic compartment by bacteria with an extracellular lifestyle remains poorly understood. One such pathogen that has been shown to activate cytosolic sensing is *Streptococcus pneumoniae* (the pneumococcus), a Gram-positive bacterium that serially colonizes the human upper respiratory tract (URT) and is a leading cause of bacterial pneumonia (1). Colonization of the upper airway precedes invasive pneumococcal disease (2) and is normally cleared by the host immune response within several weeks (3). A murine model of *S. pneumoniae* colonization has demonstrated that clearance of pneumococcal colonization requires a sustained presence of macrophages in the URT (4), similar to the observation that alveolar macrophages are critical for host defense in the lower respiratory tract (5).

The macrophage-driven clearance of colonizing pneumococci

is dependent on the host's expression of the cytosolic Nod-like receptor Nod2 (6). Recognition of the cell wall component peptidoglycan by Nod2 activates nuclear factor κ B (NF- κ B) signaling (7–9), resulting in expression of proinflammatory cytokines, including the monocyte chemoattractant protein CCL2 (or MCP-1). Macrophage recruitment to the URT, as well as subsequent bacterial clearance, is dependent on the production of CCL2 *in vivo* and *in vitro* and requires bacterial expression of the pneumococcal pore-forming toxin, pneumolysin (6).

Pneumolysin, a cholesterol-dependent cytolysin (CDC), is expressed as a monomer that oligomerizes to form a pore up to 30 nm in diameter in cholesterol-containing membranes (10). Pneumolysin is unique among the CDC family of toxins in that it lacks an N-terminal secretion signal sequence (11), but bacterial degradation by the muramidase lysozyme can cause its release in broth culture (12). Host expression of lysozyme is also required for clearance of pneumococcal colonization (6), suggesting that release of pneumolysin following lysozyme degradation allows pep-

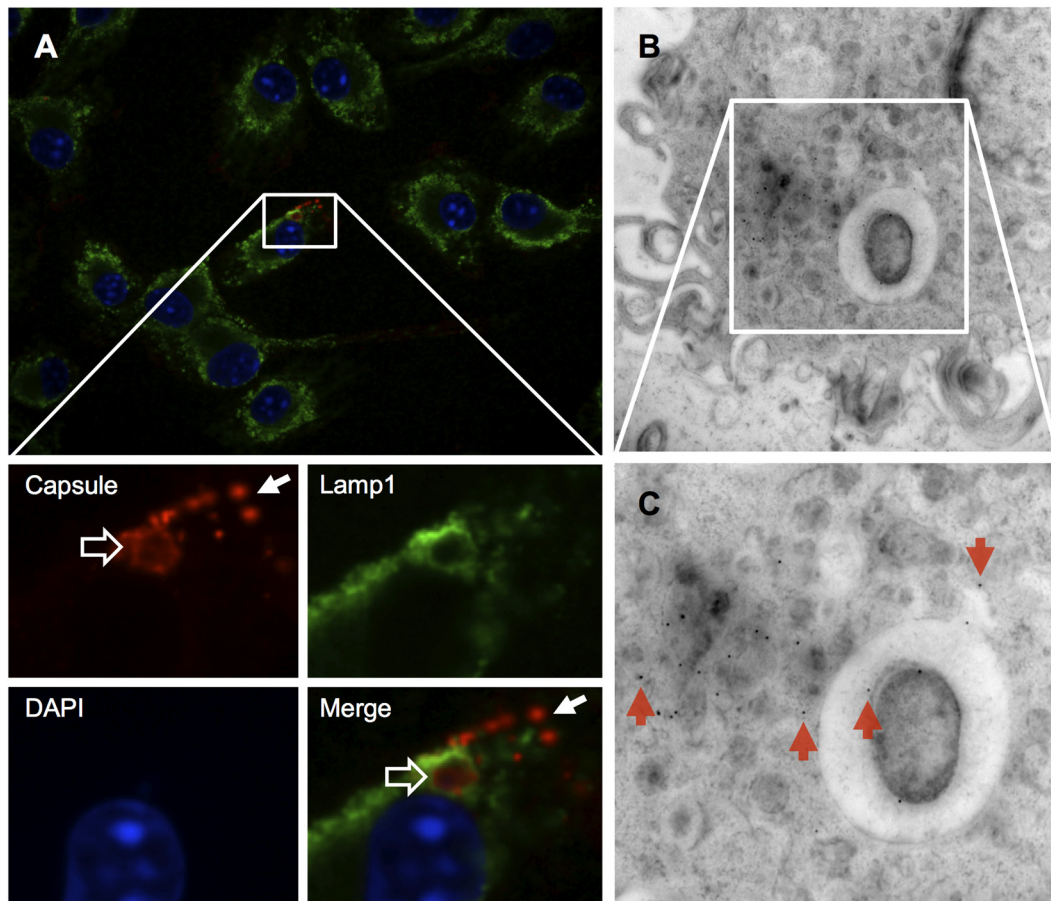


FIG 1 Pneumococcal cell wall components access the cytosol of macrophages. (A) Murine bone marrow-derived macrophages (BMMs) were incubated with *S. pneumoniae* for 2.5 h and stained for immunofluorescence with polyclonal anti-type 23F capsule sera (red, capsule), anti-Lamp1 antibody (green, phagosome), and DAPI (blue, DNA). (B and C) Electron microscopy of BMMs incubated with *S. pneumoniae* for 45 min and immuno-gold labeled with IgG purified from polyclonal anti-type 23F capsule sera. Original magnification, $\times 600$ (A), $\times 25,000$ (B), and $\times 50,000$ (C).

tidoglycan to access the host cell cytosol, where it is sensed by Nod2, eventually resulting in CCL2 production and clearance of colonization. Pneumolysin has been shown to facilitate cytosolic access of the cell wall by other bacterial species in epithelial cells for sensing by Nod1 (13), though the mechanism by which Nod2 activation occurs in phagocytes remains to be determined.

Here we show that pneumococcal cell wall-associated components escape into the cytosol of macrophages following phagocytosis and degradation. Access to the cytosol is mediated by the pore-forming toxin pneumolysin and is dependent on the ability of pneumolysin to bind host cell membranes. While the host cell has defenses to limit the amount of bacterial products that escape the phagosome, cytosolic access ultimately leads to the proinflammatory death of the macrophage.

RESULTS

Pneumococcal components access the cytosol of macrophages.

The process of cytosolic access was monitored by following the transit of the bacterial surface component capsular polysaccharide (CPS), which is covalently linked to the cell wall (14). CPS was chosen as a proxy for cell wall because it is abundant, long-lived in host cells, and easily detectable. To visualize pneumococcal fragments, we incubated murine bone marrow-derived macrophages

(BMMs) with *S. pneumoniae* and, following phagocytosis and degradation, conducted immunofluorescence staining for the pneumococcal CPS and the phagosome marker Lamp1. By confocal microscopy, we observed CPS staining within Lamp1-positive vesicles (Fig. 1A, white open arrow), as well as smaller foci of CPS that do not colocalize with the phagosome marker (Fig. 1A, white filled arrow). To address whether these fragments were in other membrane-bound organelles, we conducted immuno-gold labeling and electron microscopy of BMMs incubated with pneumococci. We observed immuno-gold staining for CPS both within the phagosome and outside any membrane-bound compartment by 45 min after infection (Fig. 1C, red arrows). At 3 h postinfection, the bacteria appeared fully degraded, but gold labeling of CPS was still visible both within and outside the phagosome (Fig. S1). Uninfected macrophages had no detectable immuno-gold labeling for CPS (data not shown). These results suggest that pneumococcal products are present in the host cell cytosol following bacterial degradation by BMMs.

The amount of pneumococcal products in the cytosol was more precisely quantified by adaptation of a detergent-based subcellular fractionation assay. Following infection with *S. pneumoniae*, BMMs were permeabilized with detergents to either lyse all host cell membranes or selectively lyse the plasma membrane.

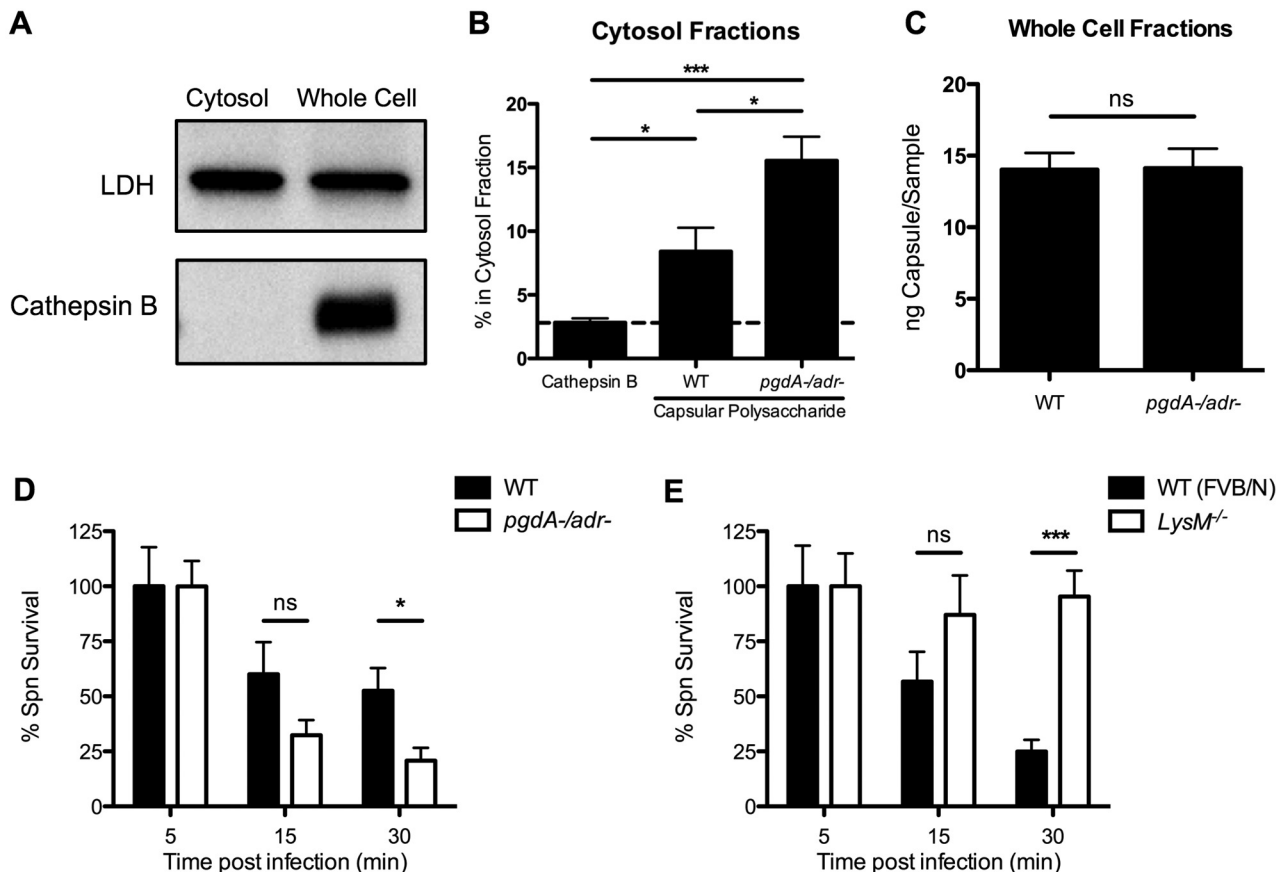


FIG 2 Quantification of pneumococcal products by cellular fractionation. (A) Western blot of subcellular fractions from bone marrow-derived macrophages (BMMs) detecting cytosolic (LDH) and lysosome (cathepsin B) protein. (B and C) Measurement of contents of cytosol fractions as a proportion of whole-cell contents by ELISA. The purity of fractions was measured by cathepsin B ELISA, with the dashed line indicating the average level of contamination of cytosolic fractions. Quantification of capsular polysaccharide (CPS) in the cytosolic fractions (B) or whole-cell fractions (C) of BMMs infected with wild-type (WT) or lysozyme-hypersensitive (*pgdA-adr*) *S. pneumoniae*. Values are from 2 to 5 independent experiments, and error bars represent \pm standard errors of the means (SEM). By one-way analysis of variance (ANOVA) with the Newman-Keuls posttest, P was <0.05 (*) and <0.001 (***). ns, not significant. (D and E) Gentamicin protection assay to measure the intracellular killing of *S. pneumoniae*. BMMs were infected with *S. pneumoniae* (Spn) and treated with 300 μ g/ml gentamicin to kill extracellular bacteria. At the time points indicated, BMMs were lysed with water and serial dilutions were plated to quantify surviving *S. pneumoniae* CFU. C57BL/6 BMMs were infected with WT or *pgdA adr* *S. pneumoniae* mutant strains (D), and BMMs from FVB/N or *LysM^{-/-}* mice were infected with the lysozyme-sensitive *pgdA adr S. pneumoniae* mutant strain (E). Percentages were calculated as proportions of CFU from the 5-min time point; values are from 2 independent experiments, and error bars represent \pm SEM. By one-way ANOVA with the Newman-Keuls posttest, P was <0.05 (*) and <0.001 (***). ns, not significant.

The resulting supernatants were ultracentrifuged to generate purified fractions representing either the cytosol or whole-cell contents. The purity of these fractions was validated by Western blotting for proteins present in either the cytosol (lactate dehydrogenase [LDH]) or the lysosome (cathepsin B) (Fig. 2A), and the amount of pneumococcal CPS in each fraction was quantified by enzyme-linked immunosorbent assay (ELISA). The percentages of CPS present in the cytosol fractions were calculated as a proportion of the CPS measured in the whole-cell fractions and were significantly higher than background levels of contamination, which were determined by ELISA for the lysosome protein cathepsin B (Fig. 2B). An *S. pneumoniae* strain lacking two cell wall-modifying enzymes, making it hypersensitive to degradation by the host enzyme lysozyme (*pgdA adr* mutant) had significantly larger amounts of CPS in the cytosol fraction than a wild-type (WT) pneumococcal strain (Fig. 2B), though no difference in total bacterial uptake was observed between the two strains, as mea-

sured by CPS in the whole-cell fractions (Fig. 2C). In a gentamicin protection assay, both the WT and *pgdA adr* strains were killed over a 30 min infection, though this process was more rapid for the *pgdA adr* mutant (Fig. 2D). BMM killing of the *pgdA adr* mutant strain was largely lysozyme dependent (Fig. 2E), correlating with the lack of intracellular signaling previously observed in lysozyme-deficient BMMs (6). Since the *pgdA adr* mutant strain had significantly higher levels of CPS present in the cytosol than the WT strain, subsequent experiments used this lysozyme-sensitive *S. pneumoniae* mutant (unless otherwise specified) in order to interrogate the host and bacterial factors mediating the access of pneumococcal cell wall products to the cytosol.

Access to the cytosol is dependent on phagocytosis and pneumolysin. To address the role of bacterial uptake in the cytosolic access of pneumococcal cell wall components, we treated BMMs with cytochalasin D (CytD), an inhibitor of actin polymerization, to block phagocytosis. Following infection with *S. pneumoniae*,

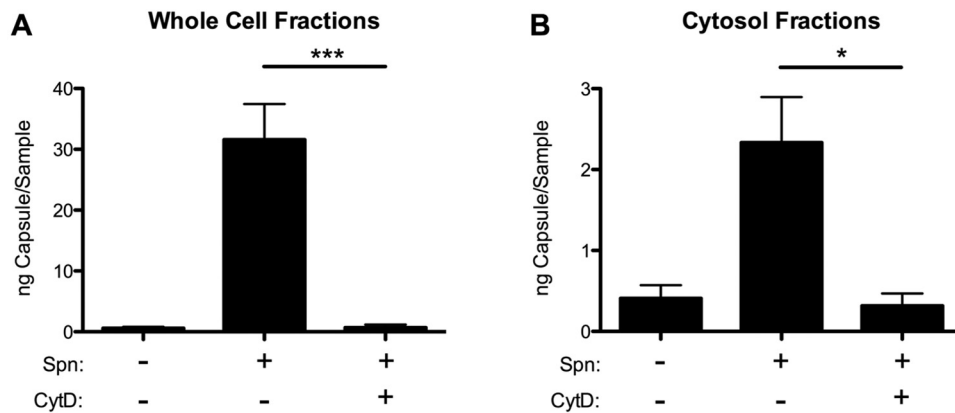


FIG 3 Access to the cytosol is dependent on bacterial uptake. (A and B) Measurement of pneumococcal capsular polysaccharide in whole-cell (A) and cytosol (B) fractions of bone marrow-derived macrophages (BMMs) by ELISA. Where indicated, BMMs were infected with lysozyme-sensitive *S. pneumoniae* (Spn) and treated with 20 μ M cytochalasin D (CytD) to block phagocytosis. Unlike in other figures, cytosolic fractions are not represented as a proportion of the whole fractions due to nearly undetectable levels of capsule in the CytD-treated whole-cell fractions. Values are from 3 independent experiments, and error bars represent \pm SEM. By one-way ANOVA with the Newman-Keuls posttest, P was <0.05 (*) and <0.001 (***)

the presence of CPS in the whole-cell fractions of host cells was blocked by incubation with CytD (Fig. 3A). The presence of CPS in the cytosol fractions upon infection with pneumococci was similarly dependent on bacterial uptake (Fig. 3B). These results demonstrate that the cytosolic access of pneumococcal CPS requires phagocytosis.

The pneumococcal pore-forming toxin, pneumolysin, is required for activation of cytosolic host signaling pathways (6). The role of pneumolysin in the cytosolic access of pneumococcal cell wall components was assessed by quantifying the amount of capsule present in the cytosol of BMMs infected with a lysozyme-sensitive *S. pneumoniae* strain containing an unmarked, complete in-frame deletion of the pneumolysin gene (*ply* mutant). The absence of pneumolysin had no effect on the ability of host cells to take up pneumococci, as seen by similar levels of CPS in the whole-cell fractions (Fig. 4A). However, compared to a bacterial strain expressing WT pneumolysin, the *ply* mutant had significantly less CPS present in the host cell cytosol (Fig. 4B). The loss of pneumolysin expression had no effect on intracellular bacterial killing (Fig. 4C). A pneumococcal strain expressing a pneumolysin toxoid containing alanine mutations in the two residues that bind cholesterol (*ply_{TL}→AA*) (15), which is deficient in pore formation as assessed by a hemolysis assay (see Fig. S2B in the supplemental material) but has no defect in the amount of pneumolysin protein that it expresses (Fig. S2B), similarly had significantly reduced levels of CPS in the cytosol fraction (Fig. 4B). Reintroduction of the pneumolysin gene to correct the mutation (*ply*⁺) fully restored the presence of CPS in the host cell cytosol (Fig. 4B). Together, these data show that pneumolysin and, specifically, toxin binding of the host membrane are required for the cytosolic access of pneumococcal cell wall components.

Cytosolic access is independent of TLR4 and acidification and is attenuated by p38 MAPK activation. Toll-like receptor 4 (TLR4) has been reported to aid in host defense through detection of pneumolysin (16). To determine the role of TLR4 in the cytosolic access of pneumococcal products, we differentiated BMMs from C57BL/6 (WT) and *Tlr4*^{-/-} mice, infected them with pneumococci, and quantified the presence of CPS by fractionation and ELISA. We observed no difference between the amounts of total

bacterial capsule in the *Tlr4*^{-/-} macrophages and the WT macrophages (Fig. 5A), and there was no significant difference in the amounts of CPS in the cytosolic fractions between WT and *Tlr4*^{-/-} macrophages (Fig. 5B). Cytosolic access, therefore, occurs independently of TLR4 activation.

Acidification of the phagosome occurs rapidly following bacterial uptake. Whether phagosomal acidification is required for bacterial products to escape into the cytosol was ascertained by incubating BMMs with bafilomycin A (BAF), a specific inhibitor of the vacuolar ATPase. The ability of BAF to block acidification was validated by Western blotting for a lysosomal protease, which matures only at low pH (Fig. S3). Measurements of CPS showed no difference in total bacterial uptake (Fig. 5C) or amount of capsule in the cytosol (Fig. 5D) between macrophages treated with BAF and untreated BMMs. These results show that the cytosolic access of pneumococcal components is independent of phagosomal acidification.

p38 mitogen-activated protein kinase (MAPK) is an important factor in host defense against diverse bacterial pore-forming toxins (17–19) and is activated in a pneumolysin-dependent manner in both epithelial cells (20) and macrophages (21). To investigate whether p38 MAPK detection of pneumolysin affects cytosolic access, we treated BMMs with SB203580, an inhibitor of p38 MAPK activation (MAPKi), before infection with *S. pneumoniae* and subcellular fractionation. When p38 MAPK was inhibited in BMMs infected with *S. pneumoniae*, we observed a significant increase in the amount of CPS in the cytosol fraction (Fig. 5F) but no change in total capsule in the whole-cell fraction (Fig. 5E). Infection of MAPKi-treated BMMs with the *ply* mutant strain showed no such increase in cytosolic CPS amounts, demonstrating that the hypersensitization observed during p38 MAPK inhibition is dependent on bacterial expression of pneumolysin (Fig. 5F). These results suggest that pneumolysin-dependent activation of p38 MAPK limits the amount of pneumococcal CPS that accesses the cytosol during infection.

Cytosolic access results in macrophage death. The final fate of host cells following infection with *S. pneumoniae* was ascertained by incubating BMMs with a lysozyme-sensitive (*pgdA* *adr* mutant) bacterial strain expressing pneumolysin or an isogenic *ply*

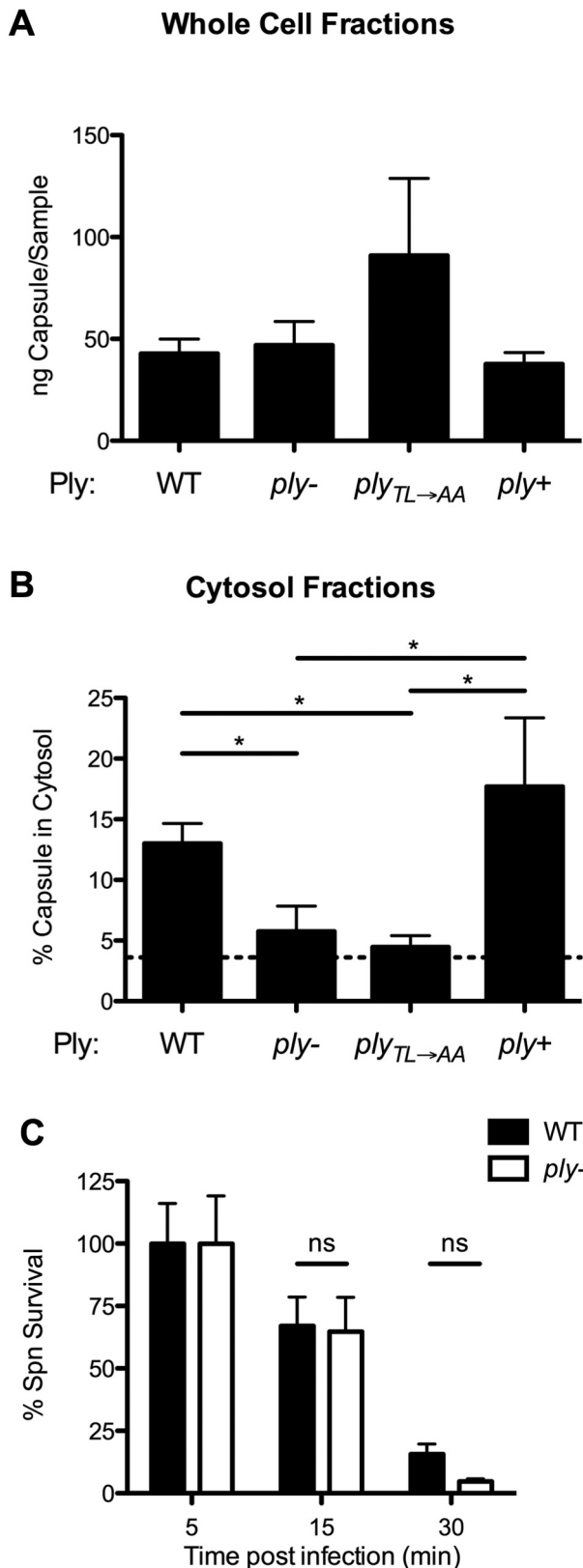


FIG 4 Pneumolysin is required for access to the host cell cytosol. (A and B) Measurement of pneumococcal capsule in whole-cell (A) and cytosol (B) fractions of bone marrow-derived macrophages (BMMs) by ELISA. Where indicated, BMMs were infected with *S. pneumoniae* strains with wild-type (WT) (Continued)

mutant strain (*pgdA adr ply*), in the presence or absence of CytD, and measuring cytotoxicity by release of LDH at 24 h after infection. BMMs infected with the pneumolysin-expressing mutant had significantly higher levels of cytotoxicity than BMMs infected with the isogenic *ply* mutant strain or those treated with CytD (Fig. 6A). These results were also observed upon infection of BMMs with the WT and its isogenic *ply* mutant bacterial strains (Fig. 6B). Addition of the p38 MAPK inhibitor prior to infection did not significantly alter macrophage cytotoxicity at 24 h postinfection (Fig. S4). These data show that macrophage death occurs following pneumococcal infection and is dependent on bacterial uptake and expression of pneumolysin.

DISCUSSION

Many Gram-positive pathogens express members of the CDC family or other pore-forming toxins (22). These toxins have diverse functions, including translocation of effectors by, for example, streptolysin O of *Streptococcus pyogenes* (23) or access to a replicative niche, as in the case of listeriolysin O (LLO) of *Listeria monocytogenes* (24). Unlike with LLO, which allows viable bacteria to access the cytosol of host cells, with *S. pneumoniae*, it is bacterial contents following degradation and killing that transit into the cytosol.

We (6) and others (25) have previously characterized Nod2-dependent intracellular sensing of extracellular pathogens. In this study, we adapted a subcellular fractionation assay, coupled with detection of CPS by ELISA, to elucidate the mechanism by which cell wall components of the extracellular bacterium *S. pneumoniae* escape into the host cell cytosol. Here, we visually and quantitatively demonstrate the transit of pneumococcal products in a manner that is dependent on lysozyme, bacterial uptake, pneumolysin, and specifically the ability of pneumolysin to bind and form pores in host membranes. Our results suggest a model in which *S. pneumoniae* is phagocytosed and degraded in the phagosome by lysozyme, which results in the release of the pore-forming toxin pneumolysin and allows the transit of bacterial components across the phagosome membrane to the cytosol, where they can be detected by intracellular host receptors. Others have recently observed phagosome disruption at a later time point postinfection, which is independent of pore formation by pneumolysin (26), suggesting that there may be more than one mechanism of accessing the host cell cytosol.

Lysozyme is abundantly expressed on both the mucosal surface (27) and within professional phagocytes (28). As a result, mucosal pathogens, including *S. pneumoniae* and *L. monocytogenes*, modify

Figure Legend Continued

pneumolysin (Ply), a complete deletion of the *ply* gene (*ply*⁻), a toxoid mutant Ply unable to bind cholesterol (*ply*_{TL→AA}), and a reintroduced fully WT Ply gene (*ply*⁺). The dashed line indicates the average level of contamination of cytosolic fractions (B). Values are from 3 to 5 independent experiments, and error bars represent \pm SEM. By one-way ANOVA with the Newman-Keuls posttest, *P* was <0.05 (*). (C) Gentamicin protection assay to measure the intracellular killing of *S. pneumoniae* (Spn). BMMs were infected with lysozyme-sensitive *S. pneumoniae* strains either deficient for pneumolysin (*ply*⁻) or expressing wild-type pneumolysin (WT). BMMs were treated with 300 μ g/ml gentamicin to kill extracellular bacteria and, at the time points indicated, were lysed with water, and serial dilutions were plated to quantify surviving *S. pneumoniae* CFU. Percentages were calculated as a proportion of CFU from the 5-min time point; values are from 2 independent experiments. ns, not significant (Student's *t* test).

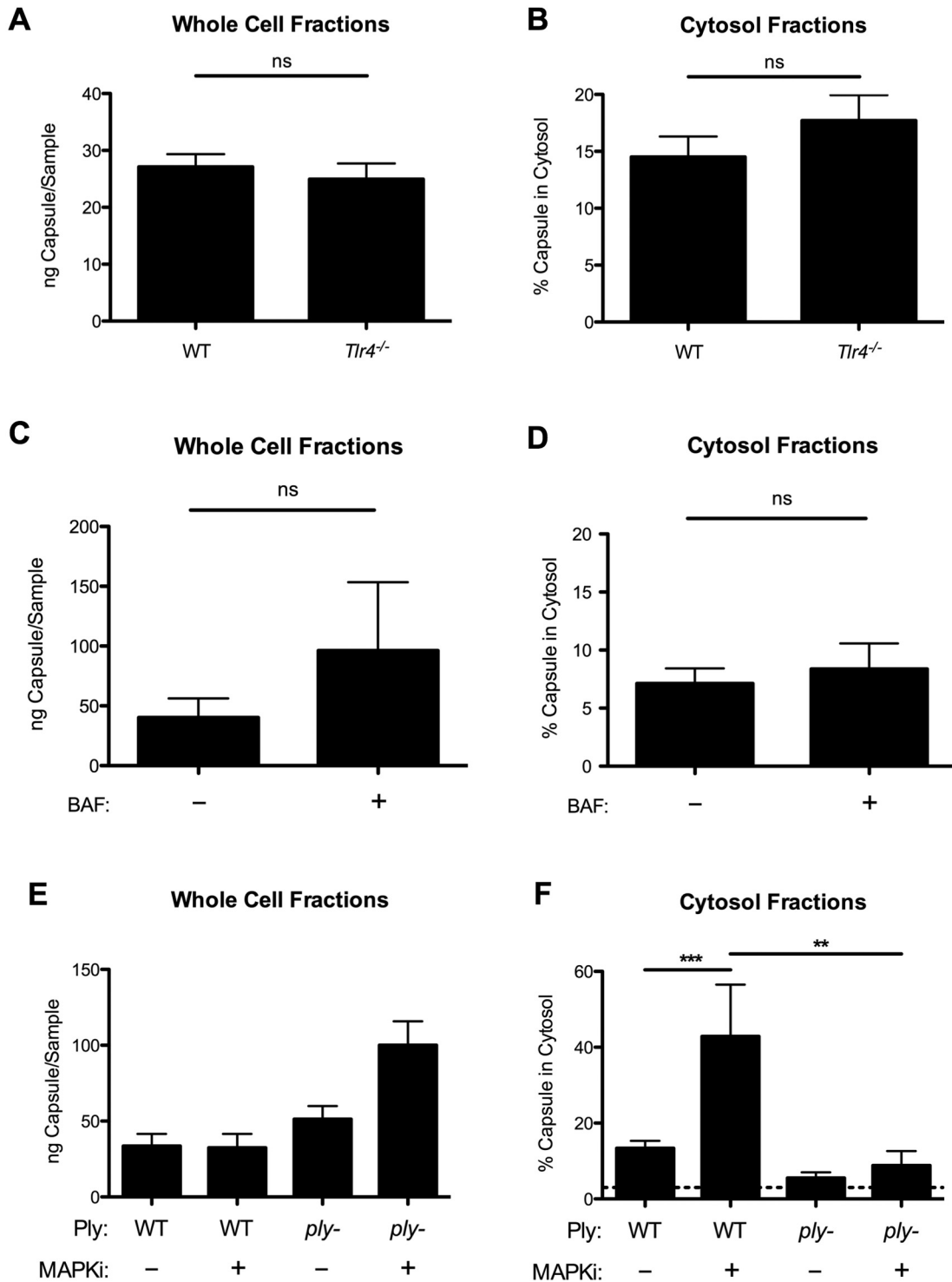


FIG 5 Cytosolic access is independent of TLR4 and acidification and is attenuated by p38 MAPK activation. (A and B) Bone marrow-derived macrophages (BMMs) from C57BL/6 (WT) and *Tlr4*^{-/-} mice were infected with *S. pneumoniae* and fractionated. Bacterial capsular polysaccharide (CPS) in the whole-cell (A) and cytosol (B) fractions was measured by ELISA. (C and D) BMMs from WT mice were treated with 30 nM baflomycin A (BAF) prior to infection with *S. pneumoniae* and subcellular fractionation. CPS in the whole-cell (C) and cytosol (D) fractions was measured by ELISA. Values are from 3 independent experiments, and error bars represent \pm SEM. ns, not significant (Student's *t* test). (E and F) BMMs were infected with an *S. pneumoniae* strain expressing pneumolysin (WT) or a pneumolysin-deficient pneumococcal strain (*ply*⁻). Where indicated, BMMs were treated with SB203580, a specific inhibitor of p38 MAPK (MAPKi). Infected BMMs were fractionated, and CPS in the whole-cell (E) and cytosol (F) fractions was quantified by ELISA. The dashed line indicates the average level of contamination of cytosolic fractions. Values are from 3 to 5 independent experiments, and error bars represent \pm SEM. By one-way ANOVA with the Newman-Keuls posttest, *P* values were <0.01 (**) and <0.001 (***).

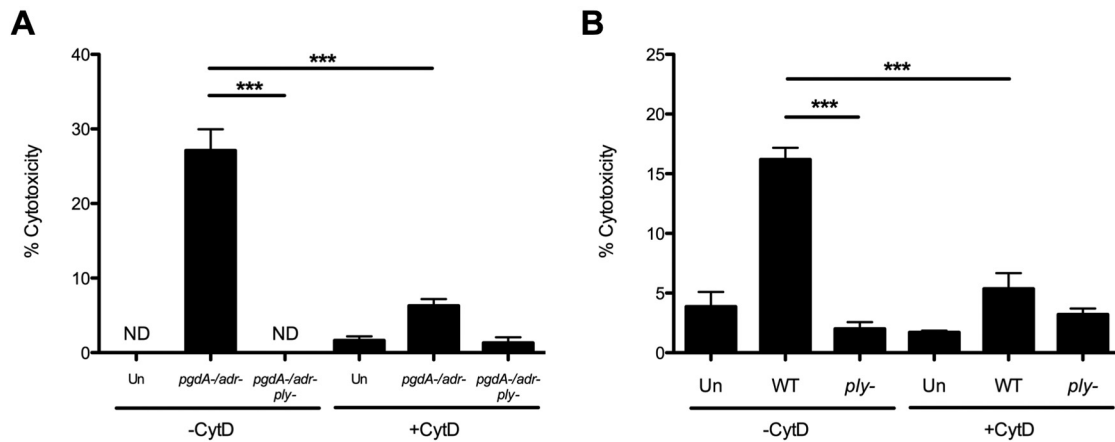


FIG 6 Cytosolic access results in macrophage death. (A and B) Bone marrow-derived macrophages (BMMs) were infected with *S. pneumoniae* strains or left uninfected (Un). Where indicated, BMMs were treated with 20 μ M cytochalasin D (CytD) to block phagocytosis. Supernatants were collected at 24 h postinfection. Cytotoxicity was determined by measuring the release of lactate dehydrogenase (LDH) in supernatants. BMMs were infected with a lysozyme-sensitive (*pgdA*⁻/*adr*⁻) and an isogenic pneumolysin-deficient (*pgdA*⁻/*adr*⁻/*ply*⁻) strain (A) or with the wild-type (WT) and a pneumolysin-deficient (*ply*⁻) strain (B). Values are from 2 to 4 independent experiments, and error bars represent \pm SEM. By one-way ANOVA with the Newman-Keuls posttest, P was <0.001 (***). ND, not detected.

their peptidoglycan to confer increased resistance to this degradative enzyme (12, 29). However, these modifications confer only partial resistance because, complementarily to previously observed *in vitro* data (12), pneumococcal killing by macrophages *ex vivo* is dependent on lysozyme, and modification to the bacterial cell wall delays, but does not block, bacterial killing. Our observation that cytosolic access does not require acidification of the phagosome is consistent with lysozyme-dependent pneumococcal degradation, which can occur in broth culture at a neutral pH (12).

Previous work has characterized a role for pneumolysin in the transit of other bacterial species' peptidoglycan across the plasma membrane of epithelial cells (13). While it is possible that lysed extracellular pneumococci access the host cytosol in a similar fashion, our finding that access to the cytosol in professional phagocytes requires bacterial uptake and degradation suggests that the transit of pneumococcal cell wall components that we have observed occurs across the phagosome membrane, rather than the plasma membrane. Although we have not directly visualized bacterial contents passing through the pore made by pneumolysin, our results demonstrate that the presence of pneumococcal products in the cytosol requires both toxin expression and function.

P38 MAPK senses a diversity of bacterial toxins, including proaerolysin (18) and streptolysin O (20), a cytolysin in the same toxin family as pneumolysin, and plays an important role in host defense. Activation of p38 MAPK occurs rapidly upon incubation of *S. pneumoniae* with macrophages (21), making it likely that initial sensing occurs at the plasma membrane prior to phagocytosis. Furthermore, purified pneumolysin is able to activate p38 MAPK (20), suggesting that detection of toxin activity is independent of host pattern recognition receptors present at the plasma membrane. Our data show that host cell sensing of pneumolysin by p38 MAPK is able to limit the transit of bacterial components to the cytosol, raising the possibility that MAPK activation attenuates phagosomal damage. Similar observations have been made for *Staphylococcus aureus* alpha-toxin, with which p38 MAPK activation is required for cellular recovery, presumably through

plasma membrane resealing (19). Lysosomal proteases have been shown to cleave pneumolysin (30), raising the possibility that host proteolysis of this pore-forming toxin may be another mechanism to limit cytosolic access. Ultimately in the case of *S. pneumoniae*, these defenses are insufficient and cytosolic access proves fatal for the host cell.

The observation that host cell death is dependent on bacterial uptake and pneumolysin raises the possibility that accessing the cytosol evolved as a mechanism to ensure the death of the host cell that takes up the organism. In the context of nasopharyngeal colonization, this may allow the pneumococcus to persist by limiting further clearance by the phagocytes that are killed. However, accessing the host cell cytosol results in Nod2-dependent proinflammatory signaling, leading to further recruitment of phagocytic cells that aid in bacterial clearance. In addition to activating Nod2, *S. pneumoniae* is known to activate type I interferon signaling pathways (31) and the cytosolic NLRP3 and AIM2 inflammasomes (32), though strain-to-strain variation has been observed (33). In these studies, host cell death was dependent on both pneumolysin expression and bacterial uptake, making it likely that the cell death observed in our study is a result of inflammasome activation. Toxin-dependent activation of the NLRP3 inflammasome and subsequent phagocyte death has also been described for other extracellular pathogens (34). While inflammasome signaling is important for host defense against pneumococcal pneumonia (32, 33, 35), the role of inflammatory cytokines, such as interleukin 1 β (IL-1 β), in the nasopharyngeal colonization of *S. pneumoniae* remains to be defined.

Intracellular bacterial pathogens access the cytosol as part of their life cycle through use of pore-forming toxins or specialized secretion systems (36). These virulence activities, however, expose pathogen-associated molecular patterns to host receptors. Here, we show that components of *S. pneumoniae*, a leading extracellular pathogen, access the cytosol via its pore-forming toxin, pneumolysin, following degradation and killing in the phagosome. Our results provide an example for how cytosolic access, and subse-

quent intracellular innate immune detection, is relevant to a clinically important extracellular microbe.

MATERIALS and METHODS

Bacterial strains and mutants. *S. pneumoniae* strains were grown in tryptic soy (TS) broth in a nonshaking water bath at 37°C to mid-log phase (optical density [OD] \approx 0.5). Mutations in *pgdA* and *adr* (12) were introduced into previously described *S. pneumoniae* strains that were either deficient in or corrected for pneumolysin expression (6, 37) by transformation with chromosomal DNA, followed by selection for kanamycin (500 μ g/ml) and spectinomycin (200 μ g/ml) resistance, respectively. Sensitivity to lysozyme was phenotypically confirmed by *in vitro* bacterial lysis as previously described (12). The *ply*_{TL \rightarrow AA} mutation, containing previously characterized alanine mutations at the threonine and leucine residues responsible for binding cholesterol (15), was constructed using overlap extension PCR with primer pairs to introduce the two alanine mutations (forward, 5'-TGG GGA ACA GCT GCC TAT CCT CAG GTA GAG GAT-3', and reverse, 5'-ATC CTC TAC CTG AGG ATA GGC AGC TGT TCC CCA-3') and primers flanking the pneumolysin gene (forward, 5'-AAA AAA GAA GCC GAT AAG GAA AAG ATG AGC G-3', and reverse, 5'-GAA AGT TTC AGC CAA GTT TGA CAA AGT CAG CTC-3'). The amplified construct was introduced into a 23F pneumococcal strain containing a Janus cassette (38) in place of the pneumolysin gene by transformation and homologous recombination. Transformants were selected for streptomycin resistance (200 μ g/ml), and sensitivity to kanamycin (200 μ g/ml) was confirmed by patching.

Hemolysis assay. As previously described (20), pellets of wild type (WT), pneumolysin-deficient (*ply*), pneumolysin toxoid (*ply*_{TL \rightarrow AA}), or corrected (*ply*⁺) *S. pneumoniae* cultures were lysed in 400 μ l lysis buffer (0.01% sodium dodecyl sulfate, 0.1% sodium deoxycholate, and 0.015 M sodium citrate) and incubated at 37°C for 30 min. Lysates were then transferred to a 96-well V-bottom plate and serially diluted 3-fold in DTT buffer (10 mM dithiothreitol, 0.1% bovine serum albumin in phosphate-buffered saline [PBS]). A 2% solution of horse red blood cells was added to each well and incubated at 37°C for 30 min. Plates were centrifuged for 10 min at 3,000 rpm to pellet unlysed cells and imaged.

Cell culture. Bone marrow cells harvested from the femurs and tibiae of C57BL/6, FVB/N, *LysM*^{-/-}, and *Tlr4*^{-/-} mice were differentiated into macrophages by culturing them in Dulbecco's modified Eagle's medium (DMEM) supplemented with 30% L929 supernatant and 20% fetal bovine serum (FBS) at 37°C with 5% CO₂ for 7 to 9 days. All animal experiments were conducted according to the guidelines outlined by National Science Foundation Animal Welfare Requirements and the Public Health Service Policy on the Humane Care and Use of Laboratory Animals. The protocol was approved by the Institutional Animal Care and Use Committee, University of Pennsylvania Animal Welfare Assurance no. A3079-01, protocol no. 803231.

Immunofluorescence staining and confocal microscopy. BMMs were seeded on to poly-L-lysine-coated coverslips (BD) in a 24-well plate at a density of 2.5×10^5 cells/slip, and the following day, they were incubated with nonopsonized *pgdA adr* mutant pneumococci at a multiplicity of infection (MOI) of 10 for 2.5 h, washed with PBS, and fixed in 3% paraformaldehyde. Cells were quenched with 50 mM NH₄Cl and permeabilized with PGS (0.01% saponin, 0.25% gelatin, and 0.02% sodium azide in PBS). To detect pneumococcal capsular polysaccharide (CPS), the cells were incubated with type 23F rabbit serum (Statens Serum Institut) at a dilution of 1:5,000 and detected with an anti-rabbit-Cy3 secondary antibody (Jackson ImmunoResearch) at a dilution of 1:600 in PGS. Phagosome membranes were labeled with a rat anti-Lamp1 antibody (eBioscience) at a dilution of 1:500 in PGS, and primary antibody was detected with an anti-rat Cy2-conjugated secondary antibody (Jackson ImmunoResearch) at a dilution of 1:600 in PGS. Nuclei were stained with 0.5 μ g/ml DAPI (4',6-diamidino-2-phenylindole) for 3 min. Slides were imaged on a Nikon Eclipse Ti-U spinning-disk confocal microscope at the Molecular Pathology and Imaging Core at the University of Pennsylvania.

Electron microscopy. Macrophage samples were high-pressure frozen in an Abra HPM-010 machine and freeze-substituted in 99% acetone, 0.1% uranyl acetate, 1% distilled water (dH₂O) in a Leica AFSII automatic freeze-substitution device. Freeze substitution was performed at -90°C for 72 h and then ramped to -50°C over 24 h, at which point cells were placed in 4-h-graded steps of 25%, 50%, 75%, 100% HM-20 in acetone. HM-20 was polymerized with 360-nm light for 48 h at -50°C and an additional 24 h at room temperature. Polymerized blocks trimmed to regions of interest were cut at 60- to 80-nm thicknesses and immunologically probed with IgG-purified type 23F rabbit serum. Protein A conjugated to 15-nm colloidal gold beads was used to secondarily detect the presence of antibody-antigen complexes. Imaging was performed on a JEOL 1010 transmission electron microscope (TEM) operating at 80 keV.

Cellular fractionation. BMMs were seeded in a 6-well, non-tissue culture-treated plate at a density of 1×10^6 cells/well. The following day, the cells were infected with nonopsonized pneumococcal strains at an MOI of 50 and spun for 5 min at 3,000 rpm. Where indicated, the BMMs were incubated with 20 μ M cytochalasin D (Sigma), 30 nM baflomycin A (Sigma), or 10 μ M SB203580 (Cell Signaling) at 37°C for 1 h prior to infection. Following infection, the cells were incubated on ice for 1 h, washed five times with PBS, and incubated for an additional 2 h at 37°C. BMMs were washed three times with PBS, lifted with PBS containing 2 mM EDTA, and permeabilized in 20 μ M digitonin (Sigma) to lyse the plasma membrane to generate cytosol fractions or 0.1% saponin (Fluka) to lyse all membranes to generate whole-cell fractions. Cells were spun at $15,000 \times g$ for 10 min, and the resulting supernatants were ultracentrifuged at 4°C for 1 h at $355,000 \times g$. Supernatants were lyophilized and resuspended in dH₂O.

Gentamicin protection assay. BMMs were seeded in a 12-well plate at a density of 4×10^5 cells/well, and the following day, they were infected with pneumococcal strains at an MOI of 50 and spun for 5 min at 3,000 rpm. The BMMs were incubated on ice for 1 h and then washed three times with PBS and incubated at 37°C for 15 min for bacterial uptake. Three hundred micrograms of gentamicin per milliliter was then added to kill remaining extracellular bacteria. At the time points indicated in the figures, the cells were lysed with dH₂O and serially diluted in PBS. Dilutions were plated on TS agar plates and incubated overnight at 37°C and 5% CO₂.

Western blotting. Subcellular fractions from BMMs were validated by running resuspended supernatants on a 10% Tris-SDS gel (Bio-Rad). Monoclonal anti-lactate dehydrogenase and polyclonal anti-cathepsin B antibodies (Abcam) were used for primary detection. Lysates from *S. pneumoniae* strains expressing pneumolysin mutations were separated on a 10% Tris-SDS gel and detected using a mouse monoclonal antipneumolysin primary antibody (Leica) and previously described (39) anti-pneumococcal surface protein A (PspA) mouse serum. Rabbit horseradish peroxidase (HRP)-conjugated (GE Healthcare) and mouse HRP-conjugated (GE Healthcare) antibodies were used for detection of the primary antibodies.

Cytotoxicity assays. BMMs were seeded in a 48-well plate at a density of 2.5×10^5 cells/well and cultured overnight at 37°C. The following day, the cells were primed with 400 ng/ml Pam3CSK4 for 4 h and then infected with *S. pneumoniae* at an MOI of 10. At 2 h postinfection, the cell culture medium was replaced with DMEM containing 300 μ g/ml gentamicin. Supernatants from infected macrophages were collected at 24 h postinfection. Lactate dehydrogenase (LDH) release was quantified using the cytotoxicity detection kit plus (Roche) per the instructions of the manufacturer.

ELISA. Subcellular fractions of infected BMMs were assayed for pneumococcal CPS by capture ELISA. Immulon 2HB plates (Thermo Scientific) were coated with type 23F rabbit serum at a dilution of 1:5,000 and incubated overnight at room temperature (RT). The plate was incubated with serial dilutions of samples or a type 23F CPS standard (American Type Culture Collection) for 2 h at RT. Samples and standards were detected with a monoclonal anti-23F CPS antibody at a 1:300 dilution for 2 h

at RT. Monoclonal antibody was detected with a goat anti-mouse alkaline phosphatase antibody (Sigma) at a 1:10,000 dilution for 2 h at RT. The plate was developed with phosphatase substrate (Sigma) for 30 min and read at an OD of 415 nm.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02110-14/-DCSupplemental>.

Figure S1, TIF file, 1.3 MB.

Figure S2, TIF file, 0.9 MB.

Figure S3, TIF file, 1.5 MB.

Figure S4, TIF file, 0.1 MB.

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REFERENCES

- O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, Lee E, Mulholland K, Levine OS, Cherian T, the Hib and Pneumococcal Global Burden of Disease Study Team. 2009. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. *Lancet* 374:893–902. [http://dx.doi.org/10.1016/S0140-6736\(09\)61204-6](http://dx.doi.org/10.1016/S0140-6736(09)61204-6).
- Bogaert D, de Groot R, Hermans PW. 2004. *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. *Lancet Infect Dis* 4:144–154. [http://dx.doi.org/10.1016/S1473-3099\(04\)00938-7](http://dx.doi.org/10.1016/S1473-3099(04)00938-7).
- McCool TL, Cate TR, Moy G, Weiser JN. 2002. The immune response to pneumococcal proteins during experimental human carriage. *J Exp Med* 195:359–365. <http://dx.doi.org/10.1084/jem.20011576>.
- Zhang Z, Clarke TB, Weiser JN. 2009. Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. *J Clin Invest* 119:1899–1909. <http://dx.doi.org/10.1172/JCI36731>.
- Knapp S, Leemans JC, Florquin S, Branger J, Maris NA, Pater J, van Rooijen N, van der Poll T. 2003. Alveolar macrophages have a protective antiinflammatory role during murine pneumococcal pneumonia. *Am J Respir Crit Care Med* 167:171–179. <http://dx.doi.org/10.1164/rccm.200207-698OC>.
- Davis KM, Nakamura S, Weiser JN. 2011. Nod2 sensing of lysozyme-digested peptidoglycan promotes macrophage recruitment and clearance of *S. pneumoniae* colonization in mice. *J Clin Invest* 121:3666–3676. <http://dx.doi.org/10.1172/JCI57761>.
- Girardin SE, Boneca IG, Viala J, Chamaillard M, Labigne A, Thomas G, Philpott DJ, Sansonetti PJ. 2003. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J Biol Chem* 278:8869–8872. <http://dx.doi.org/10.1074/jbc.C200651200>.
- Ogura Y, Inohara N, Benito A, Chen FF, Yamaoka S, Nunez G. 2001. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. *J Biol Chem* 276:4812–4818. <http://dx.doi.org/10.1074/jbc.M008072200>.
- Tanabe T, Chamaillard M, Ogura Y, Zhu L, Qiu S, Masumoto J, Ghosh P, Moran A, Predergast MM, Tromp G, Williams CJ, Inohara N, Nunez G. 2004. Regulatory regions and critical residues of NOD2 involved in muramyl dipeptide recognition. *EMBO J* 23:1587–1597. <http://dx.doi.org/10.1038/sj.emboj.7600175>.
- Korchev YE, Bashford CL, Pederzoli C, Pasternak CA, Morgan PJ, Andrew PW, Mitchell TJ. 1998. A conserved tryptophan in pneumolysin is a determinant of the characteristics of channels formed by pneumolysin in cells and planar lipid bilayers. *Biochem J* 329:571–577.
- Walker JA, Allen RL, Falmagne P, Johnson MK, Boulnois GJ. 1987. Molecular cloning, characterization, and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of *Streptococcus pneumoniae*. *Infect Immun* 55:1184–1189.
- Davis KM, Akinbi HT, Standish AJ, Weiser JN. 2008. Resistance to mucosal lysozyme compensates for the fitness deficit of peptidoglycan modifications by *Streptococcus pneumoniae*. *PLoS Pathog* 4:e1000241. <http://dx.doi.org/10.1371/journal.ppat.1000241>.
- Ratner AJ, Aguilar JL, Shchepetov M, Lysenko ES, Weiser JN. 2007. Nod1 mediates cytoplasmic sensing of combinations of extracellular bacteria. *Cell Microbiol* 9:1343–1351. <http://dx.doi.org/10.1111/j.1462-5822.2006.00878.x>.
- Sørensen UB, Henrichsen J, Chen HC, Szu SC. 1990. Covalent linkage between the capsular polysaccharide and the cell wall peptidoglycan of *Streptococcus pneumoniae* revealed by immunochemical methods. *Microb Pathog* 8:325–334. [http://dx.doi.org/10.1016/0882-4010\(90\)90091-4](http://dx.doi.org/10.1016/0882-4010(90)90091-4).
- Farrand AJ, LaChapelle S, Hotze EM, Johnson AE, Tweten RK. 2010. Only two amino acids are essential for cytolytic toxin recognition of cholesterol at the membrane surface. *Proc Natl Acad Sci U S A* 107:4341–4346. <http://dx.doi.org/10.1073/pnas.0911581107>.
- Malley R, Henneke P, Morse SC, Cieslewicz MJ, Lipsitch M, Thompson CM, Kurt-Jones E, Paton JC, Wessels MR, Golenbock DT. 2003. Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *Proc Natl Acad Sci U S A* 100:1966–1971. <http://dx.doi.org/10.1073/pnas.0435928100>.
- Nagahama M, Shibutani M, Seike S, Yonezaki M, Takagishi T, Oda M, Kobayashi K, Sakurai J. 2013. The p38 MAPK and JNK pathways protect host cells against *Clostridium perfringens* beta-toxin. *Infect Immun* 81:3703–3708. <http://dx.doi.org/10.1128/IAI.00579-13>.
- Huffman DL, Abrami L, Sasik R, Corbeil J, van der Goot FG, Aroian RV. 2004. Mitogen-activated protein kinase pathways defend against bacterial pore-forming toxins. *Proc Natl Acad Sci U S A* 101:10995–11000. <http://dx.doi.org/10.1073/pnas.0404073101>.
- Husmann M, Dersch K, Bobkiewicz W, Beckmann E, Veerachato G, Bhakdi S. 2006. Differential role of p38 mitogen activated protein kinase for cellular recovery from attack by pore-forming *S. aureus* alpha-toxin or streptolysin O. *Biochem Biophys Res Commun* 344:1128–1134. <http://dx.doi.org/10.1016/j.bbrc.2006.03.241>.
- Ratner AJ, Hippe KR, Aguilar JL, Bender MH, Nelson AL, Weiser JN. 2006. Epithelial cells are sensitive detectors of bacterial pore-forming toxins. *J Biol Chem* 281:12994–12998. <http://dx.doi.org/10.1074/jbc.M511431200>.
- Das R, LaRose MI, Hergott CB, Leng L, Bucala R, Weiser JN. 2014. Macrophage migration inhibitory factor promotes clearance of pneumococcal colonization. *J Immunol* 193:764–772. <http://dx.doi.org/10.4049/jimmunol.1400133>.
- Tweten RK. 2005. Cholesterol-dependent cytolysins, a family of versatile pore-forming toxins. *Infect Immun* 73:6199–6209. <http://dx.doi.org/10.1128/IAI.73.10.6199-6209.2005>.
- Madden JC, Ruiz N, Caparon M. 2001. Cytolysin-mediated translocation (CMT): a functional equivalent of type III secretion in Gram-positive bacteria. *Cell* 104:143–152. [http://dx.doi.org/10.1016/S0092-8674\(01\)00198-2](http://dx.doi.org/10.1016/S0092-8674(01)00198-2).
- Portnoy DA, Jacks PS, Hinrichs DJ. 1988. Role of hemolysin in the intracellular growth of *Listeria monocytogenes*. *J Exp Med* 167:1459–1471. <http://dx.doi.org/10.1084/jem.167.4.1459>.
- Hruz P, Zinkernagel AS, Jenikova G, Botwin GJ, Hugot J-P, Karin M, Nizet V, Eckmann L. 2009. NOD2 contributes to cutaneous defense against *Staphylococcus aureus* through alpha-toxin-dependent innate immune activation. *Proc Natl Acad Sci U S A* 106:12873–12878. <http://dx.doi.org/10.1073/pnas.0904958106>.
- Bewley MA, Naughton M, Preston J, Mitchell A, Holmes A, Marriott HM, Read RC, Mitchell TJ, Whyte MK, Dockrell DH. 2014. Pneumolysin activates macrophage lysosomal membrane permeabilization and executes apoptosis by distinct mechanisms without membrane pore formation. *mBio* 5(5):e01710-14. <http://dx.doi.org/10.1128/mBio.01710-14>.
- Cole AM, Liao H-I, Stuchlik O, Tilan J, Pohl J, Ganz T. 2002. Cationic polypeptides are required for antibacterial activity of human airway fluid. *J Immunol* 169:6985–6991. <http://dx.doi.org/10.4049/jimmunol.169.12.6985>.
- Faust N, Varas F, Kelly LM, Heck S, Graf T. 2000. Insertion of enhanced green fluorescent protein into the lysozyme gene creates mice with green fluorescent granulocytes and macrophages. *Blood* 96:719–726.
- Rae CS, Geissler A, Adamson PC, Portnoy DA. 2011. Mutations of the *Listeria monocytogenes* peptidoglycan N-deacetylase and O-acetylase result in enhanced lysozyme sensitivity, bacteriolysis, and hyperinduction of innate immune pathways. *Infect Immun* 79:3596–3606. <http://dx.doi.org/10.1128/IAI.00077-11>.
- Carrasco-Marín E, Madrazo-Toca F, de los Toyos JR, Cacho-Alonso E, Tobes R, Pareja E, Paradelo A, Albar JP, Chen W, Gomez-Lopez MT,

- Alvarez-Dominguez C. 2009. The innate immunity role of cathepsin-D is linked to Trp-491 and Trp-492 residues of listeriolysin O. *Mol Microbiol* 72:668–682. <http://dx.doi.org/10.1111/j.1365-2958.2009.06673.x>.
31. Parker D, Martin FJ, Soong G, Harfenist BS, Aguilar JL, Ratner AJ, Fitzgerald KA, Schindler C, Prince A. 2011. Streptococcus pneumoniae DNA initiates type I interferon signaling in the respiratory tract. *mBio* 2(3):e00016–11. <http://dx.doi.org/10.1128/mBio.00016-11>.
 32. Fang R, Tsuchiya K, Kawamura I, Shen Y, Hara H, Sakai S, Yamamoto T, Fernandes-Alnemri T, Yang R, Hernandez-Cuellar E, Dewamitta SR, Xu Y, Qu H, Alnemri ES, Mitsuyama M. 2011. Critical roles of ASC inflammasomes in caspase-1 activation and host innate resistance to Streptococcus pneumoniae infection. *J Immunol* 187:4890–4899. <http://dx.doi.org/10.4049/jimmunol.1100381>.
 33. Witznath M, Pache F, Lorenz D, Koppe U, Gutbier B, Tabeling C, Reppe K, Meixenberger K, Dorhoi A, Ma J, Holmes A, Trendelenburg G, Heimesaat MM, Bereswill S, van der Linden M, Tschopp J, Mitchell TJ, Suttrop N, Opitz B. 2011. The NLRP3 inflammasome is differentially activated by pneumolysin variants and contributes to host defense in pneumococcal pneumonia. *J Immunol* 187:434–440. <http://dx.doi.org/10.4049/jimmunol.1003143>.
 34. Gupta R, Ghosh S, Monks B, Deoliveira RB, Tzeng TC, Kalantari P, Nandy A, Bhattacharjee B, Chan J, Ferreira F, Rathinam V, Sharma S, Lien E, Silverman N, Fitzgerald K, Firon A, Trieu-Cuot P, Henneke P, Golenbock DT. 2014. RNA and β -hemolysin of group B streptococcus induce IL-1 β by activating NLRP3 inflammasomes in mouse macrophages. *J Biol Chem* 289:13701–13705. <http://dx.doi.org/10.1074/jbc.C114.548982>.
 35. Kafka D, Ling E, Feldman G, Benharroch D, Voronov E, Givon-Lavi N, Iwakura Y, Dagan R, Apte RN, Mizrahi-Nebenzahl Y. 2008. Contribution of IL-1 to resistance to Streptococcus pneumoniae infection. *Int Immunol* 20:1139–1146. <http://dx.doi.org/10.1093/intimm/dxn071>.
 36. Vance RE, Isberg RR, Portnoy DA. 2009. Patterns of pathogenesis: discrimination of pathogenic and nonpathogenic microbes by the innate immune system. *Cell Host Microbe* 6:10–21. <http://dx.doi.org/10.1016/j.chom.2009.06.007>.
 37. Matthias KA, Roche AM, Standish AJ, Shchepetov M, Weiser JN. 2008. Neutrophil-toxin interactions promote antigen delivery and mucosal clearance of Streptococcus pneumoniae. *J Immunol* 180:6246–6254. <http://dx.doi.org/10.4049/jimmunol.180.9.6246>.
 38. Sung CK, Li H, Claverys JP, Morrison DA. 2001. An rpsL cassette, Janus, for gene replacement through negative selection in Streptococcus pneumoniae. *Appl Environ Microbiol* 67:5190–5196. <http://dx.doi.org/10.1128/AEM.67.11.5190-5196.2001>.
 39. Darrieux M, Moreno AT, Ferreira DM, Pimenta FC, de Andrade AL, Lopes AP, Leite LC, Miyaji EN. 2008. Recognition of pneumococcal isolates by antisera raised against PspA fragments from different clades. *J Med Microbiol* 57:273–278. <http://dx.doi.org/10.1099/jmm.0.47661-0>.