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Neutrophil-Toxin Interactions Promote Antigen Delivery and Mucosal Clearance of *Streptococcus pneumoniae*1

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Delivery of Ag to inductive sites, such as nasal-associated lymphoid tissue (NALT) or GALT, is thought to promote mucosal immunity. Host and microbial factors that contribute to this process were investigated during model murine airway colonization by the pathogen *Streptococcus pneumoniae*. Colonization led to the deposition of released bacterial capsular Ag in the NALT in a manner consistent with trafficking through M cells. This Ag was derived from processing of bacteria in the lumen of the paranasal spaces rather than through invasion or sampling of intact bacteria. Neutrophils, which are recruited to the paranasal spaces where they associate with and may degrade bacteria, were required for efficient Ag delivery. Maximal Ag delivery to the NALT also required expression of the bacterial toxin pneumolysin. Pneumolysin and pneumolysin-expressing bacteria lysed neutrophils through pore formation in vitro. Accordingly, a pneumolysin-dependent loss of neutrophils, which correlated with the increased release of bacterial products, was observed in vivo. Thus, delivery of Ag to the NALT was enhanced by neutrophil-mediated generation of bacterial products together with bacterial-induced lysis of neutrophils. The impaired Ag delivery of pneumolysin-deficient bacteria was associated with diminished clearance from the mucosal surface. This study demonstrates how microbial-host interactions affect Ag delivery and the effectiveness of mucosal immunity. *The Journal of Immunology*, 2008, 180: 6246–6254.

Colonization of mucosal surfaces is the initial interaction with a host for many commensal and pathogenic microbes. However, this interaction may be short-lived with recognition of microbial products stimulating an immune response that leads to eventual clearance. Microbial and host factors that affect inflammation and the development of mucosal immunity during colonization remain poorly understood. Successful mucosal pathogens often express virulence factors such as toxins that inhibit the antimicrobial activity of recruited inflammatory cells (1). Microbial manipulation of these innate immune responses may, in turn, affect the generation of adaptive immunity, altering the dynamics of colonization and infection (2). This report focuses specifically on how these host-microbe interactions may affect the delivery of Ag to the mucosal immune system.

In a prior study of experimental human colonization with *Streptococcus pneumoniae* (the pneumococcus), the duration of bacterial carriage of a type 23F isolate ranged from 3 to 122 days (3). When mice were challenged intranasally with the same isolate and inoculum, the duration of colonization and the resulting immune response were similar to those of the natural human host (4). Colonization of both humans and mice resulted in the production of capsular polysaccharide-specific Abs, which was associated with reduced nasopharyngeal carriage of the type 23F isolate (3). Colonization led to the deposition of released bacterial capsular Ag in the NALT in a manner consistent with trafficking through M cells. This Ag was derived from processing of bacteria in the lumen of the paranasal spaces rather than through invasion or sampling of intact bacteria. Neutrophils, which are recruited to the paranasal spaces where they associate with and may degrade bacteria, were required for efficient Ag delivery. Maximal Ag delivery to the NALT also required expression of the bacterial toxin pneumolysin. Pneumolysin and pneumolysin-expressing bacteria lysed neutrophils through pore formation in vitro. Accordingly, a pneumolysin-dependent loss of neutrophils, which correlated with the increased release of bacterial products, was observed in vivo. Thus, delivery of Ag to the NALT was enhanced by neutrophil-mediated generation of bacterial products together with bacterial-induced lysis of neutrophils. The impaired Ag delivery of pneumolysin-deficient bacteria was associated with diminished clearance from the mucosal surface. This study demonstrates how microbial-host interactions affect Ag delivery and the effectiveness of mucosal immunity.

In the human upper respiratory tract, the tonsils and adenoids constitute a primary site of exposure to inhaled pathogens. The Waldeyer’s ring, as the tissues are collectively called, contains T and B cells and is thought to be important in adaptive immune responses to respiratory flora, including *S. pneumoniae* (9, 10). Mice lack the Waldeyer’s ring but contain the analogous nasal-associated lymphoid tissue (NALT), which extends the length of the ventral surface of the upper palate and has been implicated as an inductive site for specific T cell priming of streptococcal Ags (11–13). Park et al. proposed that T cell priming was initiated when *Streptococcus pyogenes* directly entered the NALT through M cells on the adjacent mucosal surface (14). If the NALT acts similarly as an inductive site upon pneumococcal infection, then delivery of pneumococcal Ag to the NALT should enhance bacterial clearance.

We previously demonstrated that pneumococcal colonization of the murine upper respiratory tract led to an acute inflammatory response characterized by the influx of neutrophils into the paranasal spaces (7). These neutrophils, which were observed for <7 days postinoculation, appeared to be insufficient to control initial mucosal colonization but may affect the immune response and contribute to the pathogen’s eventual clearance (15). An important determinant of the inflammatory response to the pneumococcus is its sole toxin, pneumolysin, a member of a family of thiol-activated, cholesterol-binding cytotoxins expressed by many Gram-positive pathogens. Pneumolysin has been associated with multiple effects including the stimulation of neutrophil recruitment and T cell responses (7, 16–18).

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*Abbreviations used in this paper: NALT, nasal-associated lymphoid tissue; IF, immunofluorescence; DAPI, 4',6-diamidino-2-phenylindole; Ply, purified pneumolysin; PdB, purified pneumolysin toxoid; LDH, lactate dehydrogenase; wt, wild type; UEA-1, *Ulex europaeus* agglutinin-1.*
In this report, we illustrate that the interaction of host neutrophils with the pore-forming toxin pneumolysin leads to release and delivery of pneumococcal Ag to the NALT and correlates with more rapid clearance of *S. pneumoniae* from the nasopharynx. These results demonstrate the importance of the acute inflammatory response in pneumococcal clearance, not as a direct means of controlling infection, but rather as an enhancer of adaptive immunity.

**Materials and Methods**

**Bacterial strains and culture**

*S. pneumoniae* strains were grown in tryptic soy medium as described elsewhere (19). Strain P1121, a type 23F isolate obtained from a study of experimental human colonization, was selected for its ability to efficiently colonize the murine nasopharynx (3, 4). The construction of defined mutants of P1121 using the bicistronic positively and negatively selectable Janus cassette technology was previously described and included: 1) pLy*, a strain containing an unmarked, complete in-frame deletion of the pneumolysin gene; and 2) pWu*, a strain with the deletion restored with a point mutation previously shown to reduce the efficiency of pore formation (20–23). The genotype of pLy* and pWu* was confirmed by sequencing across the introduced mutation. Expression of pneumolysin was assessed by Western blot analysis probing with anti-pneumolysin Ab (Novocastra Laboratories). A horse erythrocyte lysis assay was used to assess pore formation as previously described (24). An encapsulated type IV isolate (TIGR4) was also used in immunofluorescence (IF) (3) studies on NALT. All strains were passage intranasally in mice before preparation of frozen stocks.

**Animal colonization model**

Female BALB/c mice (5–8 wk of age) were obtained from Taconic Laboratories and housed in accordance with the protocols established by the Institutional Animal Care and Use Committee. Mice were colonized for a period of 1–25 days as previously described (4, 25). Briefly, an intranasal inoculation of 10^7 CFU (3) of PBS-washed, mid-log phase bacteria in 10 μl was administered drop-wise to the external nares. This procedure was found to be effective in achieving colonization of the upper respiratory tract. Animals were sacrificed and the tracheas cannulated. A total volume of 200 μl of PBS was instilled into the trachea and recovered from the nares for quantitative culture. Serial dilutions of upper respiratory tract lavage were plated on tryptic soy medium with catalase and neomycin (20 μg/ml) to minimize the growth of contaminants. The pLy* and pWu* mutants were plated on selective medium with streptomycin (100 μg/ml). The lower limit of detection for bacteria obtained from the nasal lavage was 20 CFU/ml.

**Neutrophil depletion**

RR6-8C5, a rat anti-mouse IgG2b Ab directed against Ly-6G on the surface of murine myeloid (and limited subpopulations of lymphoid) lineage cells, was purified from ascites of nude mice given the RB6-8C5 hybridoma (26, 27). Neutrophil depletion was achieved by i.p. delivery of 100 μg Ab per animal 24 h before intranasal inoculation. This treatment was shown in pilot experiments to result in peripheral blood neutropenia (<50 granulocytes/μl) for a period of at least 4 days. Control animals were administered the equivalent dose of total rat IgG (Sigma-Aldrich).

**Flow cytometry**

The nasal lavages of five mice from each group were pooled, centrifuged at 1500 × g, and resuspended in 1% BSA in PBS. Non-specific binding was blocked using a rat anti-mouse Ab directed against the F(ab′)2 fragment receptor (CD16/CD32) and the following Abs were applied: rat anti-mouse Ly-6G to detect neutrophils; rat anti-mouse CD11b, a cell-surface marker of neutrophil activation; hamster anti-mouse CD11c to detect dendritic cells; rat anti-mouse CD14 to detect monocytes; and rat anti-mouse F4/80 to detect macrophages. All Abs were obtained from BD Biosciences except for anti-F4/80, which was purchased from eBioscience. A total of 10,000 cells were collected for each sample and groups were compared using FlowJo software (Tree Star).

**Histology and IF**

Three days postinoculation, mice were sacrificed and decapitated. Heads were treated as described elsewhere (15). Briefly, samples were fixed for 48 h in formalin (4% paraformaldehyde) and decalcified for 30 days in 0.12 M EDTA (pH 7.0). The heads were then frozen in Tissue-Tek OCT embedding medium (Electron Microscopy Sciences) and 5-μm sections cut, and either stained with H&E or stored at −80°C for IF.

For IF labeling of bacteria, neutrophils, macrophages, and dendritic cells, all sections were postfixed in 1/1 methanol-acetone at −20°C for 10 min followed by washing in PBS. Nonspecific binding was inhibited by incubation (10 min) in protein-blocking reagent (Coulter-Immunotech). Bacteria were detected using specific antisera (diluted 1/500) recognizing the Fco23F or type 4 capsular polysaccharide (Staten Serum Institut) followed by Cy3-conjugated anti-rabbit Ig secondary Abs (diluted 1/400, Jackson Immunoresearch). Neutrophils were detected by application of anti-Ly-6G (diluted 1/500) and Cy2-conjugated anti-rabbit Igs (diluted 1/400, Jackson Immunoresearch). Dendritic cells and macrophages were detected using the same technique as that for neutrophil detection, except for route of antibody entry: primary Ab was applied in PBS, secondary Abs (Rat anti-mouse CD11c and anti-hamster-Cy2 for dendritic cells; rat anti-mouse F4/80 and anti-rat-Cy2 for macrophages). Murine cells were visualized by counterstaining with 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes-Invitrogen).

The same procedure as above was used to selectively label M cells, except postfixing was performed in acetone alone and nonspecific blocking with protein-blocking reagent was followed by application of the Avidin/ Biotin Blocking Kit according to the manufacturer’s protocol (Vector Laboratories). Bacteria were detected as described above, and sections were colabeled with 50 μg/ml of a biotinylated lectin (Ulex europaeus agglutinin-1 (UEA-1), Vector Laboratories), which has been shown to selectively label M cells in the gut and the nasal mucosa (14). UEA-1 labeling was detected with a Cy2-conjugated anti-avidin secondary Ab (Jackson Immunoresearch) and cells were counterstained with DAPI.

All IF imaging was performed on a Nikon Eclipse E600 microscope equipped with a high-resolution charge-coupled device digital camera (CoolSNAP cf, Roper Scientific). Image analysis was conducted using IPLAB software (Scanalytics).

**Gentamicin protection assay**

To determine whether viable pneumococci were present within cells, the NALT was dissected from mice as described previously after 3 days of infection with the wild-type (wt) strain (28, 29). Cells were passed through a wire mesh screen to generate a single-cell suspension and incubated with gentamicin sulfate (final concentration 300 μg/ml) at 37°C for 2 h in RPMI 1640 (Invitrogen), conditions reported in a previous study to be sufficient to kill extra- but not intracellular strain P1121 (30). The cells were spun down and washed to remove the antibiotic, and serial dilution plating was used to determine the number of viable intracellular bacteria.

**ELISA**

Nasal lavage and serum samples were tested following 25 days of bacterial infection for levels of anti-pneumococcal IgA or IgG and IgM Abs, respectively. Whole-cell wt bacteria grown on overnight cultures of tryptic soy agar were suspended in sterile phosphate-buffered saline (pH 7.2) (0.15 M NaCl, 0.05 M NaHCO3, (pH 9.6)) and used to coat 96-well Immulon 2HB plates (Thermo Electron) at an OD of 0.1. Alternatively, plates were coated with purified pneumococcal surface protein A (0.5 μg/ml). Plates were incubated overnight at 4°C, washed with 0.05% PBS-Brij, and blocked by 1 h incubation in 1% BSA (Sigma-Aldrich). After blocking, samples were plated in 2-fold serial dilutions and incubated overnight at 4°C. Samples were then washed in PBS-Brij, and Ag-specific Abs were detected by 2 h incubation (37°C) in alkaline phosphatase-conjugated goat anti-mouse IgGs (anti-IgA for lavage samples, anti-IgG/anti-IgM for serum, at a dilution of 1/4000) (Sigma-Aldrich). Plates were developed by 1 h incubation in p-nitrophenyl phosphate (Sigma-Aldrich) and absorbencies were recorded at 415 nm. Geometric mean titers were assessed by calculating the sample dilution at which A = 0.1.

**MIP-2 levels**

MIP-2 levels were measured in nasal lavage specimens using a MIP-2 Quantikine ELISA kit (R&D Systems).

**Isolation of neutrophils and phagocytic killing assay**

Neutrophil-enriched peritoneal exudate cells were isolated from BALB/c mice as previously described (31). Briefly, mice were administered two separate 1.0 ml i.p. injections of 10% casein in PBS 24 h and 2 h before cell harvest. Animals were sacrificed and phagocytes obtained by peritoneal lavage using 8 ml of 0.1% gelatin in HBSS without Mg2+ or Ca2+ (Intralipid). Phagocytes were enriched for neutrophils by separation on a Ficoll density gradient centrifugation using Mono-Poly resolving medium (MP Biomedicals). Cells were then collected and washed with 5 ml of HBSS and counted by trypan blue exclusion.
Neutrophils were adjusted to a density of $7.0 \times 10^7$ cells/ml by resuspension in HBSS containing Mg$^{2+}$ and Ca$^{2+}$ (Invitrogen) and 0.1% gelatin. PBS-washed mid-log phase bacteria (wt, ply$^-$, Ply-W433F) were opsonized by incubation in 10% fresh murine serum (used as a source of complement) in HBSS for 30 min. Bacteria were then added to neutrophils at a ratio of 1:10 (bacterium/neutrophils), and phagocytic killing was determined after a 45 min incubation at 37°C, following which time samples were collected and the reaction was stopped by incubation at 4°C. The number of remaining bacteria was assessed by serial dilution plating on selective media, and the percentage of pneumococcal survival was determined relative to control groups without neutrophils.

**Cytotoxicity assay**

Neutrophils were prepared in HBSS as described above, except that the final density of cells was adjusted to $2.5 \times 10^7$ per ml. A total of $5.0 \times 10^8$ neutrophils were plated per well of a 96-well plate and combined with either purified pneumolysin toxin (Ply) or (PdB), a pneumolysin toxoid containing the W433F point mutation that exhibits 1–5% lytic activity in bacteria 

$\text{LDH}_\text{cytotoxicity} = \frac{\text{Experimental value} \ - \ \text{Low control}}{\text{High control} \ - \ \text{Low control}} \times 100$

For flow cytometry analysis and in vitro killing assays, groups were compared by two-way ANOVA. Statistical differences in the LDH cytotoxicity assay were determined by repeated measures ANOVA with Tukey’s post-test. The Mann-Whitney U test was used to evaluate differences in colonization density, and the Kaplan-Meier log-rank test was used to compare differences in mouse survival due to neutrophil depletion. All statistics were evaluated using Prism 4 Software (GraphPad Software).

**Results**

**Role of neutrophils in Ag delivery to the NALT**

IF studies on tissue sections through the nasopharynx revealed a temporal correlation between the influx of neutrophils and the deposition of capsular polysaccharide Ag in central regions of the NALT, with both peaking between days 1 and 3 postinoculation (data not shown). Intact bacteria, which were seen in large numbers in association with neutrophils in superior and lateral para-nasal spaces, were never observed within the NALT, which is located more ventral to these collections. This suggested that Ag removal from intact bacteria was occurring outside the NALT and that neutrophils may contribute to this process. To determine whether the presence of neutrophils in the nasal spaces directly affects Ag delivery to the NALT, we rendered mice neutropenic by pretreatment with RB6-8C5, a rat mAb directed against the surface-expressed marker Ly-6G. After 3 days of infection with a type 23F clinical isolate (wt), the NALT was examined by IF for the deposition of pneumococcal-specific Ag. In comparison with controls (Fig. 1A), the NALT of neutropenic mice exhibited a dramatic decrease in the amount of NALT-associated Ag (Fig. 1B).

**Role of pneumolysin in Ag delivery to the NALT**

To address the mechanisms leading to neutrophil-mediated Ag delivery to the NALT, we focused on the contribution of the intracellular bacterial toxin pneumolysin. Because infection with bacteria expressing pneumolysin causes a robust influx of neutrophils (7, 32), we hypothesized that infecting mice with a strain deficient in pneumolysin would cause diminished Ag delivery to the NALT. Moreover, because the secretion of neutrophil-attracting chemokines from epithelial cells has been shown to be dependent on pneumolysin’s pore-forming capacity, we further hypothesized that only pneumolysin capable of forming pores would promote Ag delivery (24).

To examine these hypotheses, we constructed isogenic pneumolysin mutants of the wt strain. These included ply$^-$, a strain containing an in-frame deletion of the entire pneumolysin gene, and Ply-W433F, a strain containing a single amino acid change rendering the toxin deficient in pore formation. Lack of pneumolysin expression by ply$^-$ and equivalent expression by the wt and Ply-W433F strains were shown by Western blot (Fig. 2A). Additionally, the ply$^-$ and Ply-W433F mutants exhibited >32-fold reduction in...
the ability to form pores in host cell membranes as determined by a hemolysis assay (Fig. 2B).

After verifying the phenotype in the pneumolysin mutants, ply" and Ply-W433F were inoculated into mice and the presence of type 23F capsular Ag was assessed by IF. At 3 days postinoculation, Ag delivery to the NALT was decreased in both mutants compared with the wt parent strain and was similar to neutrophil-depleted mice infected with the wt parent strain (Fig. 1, C and D).

**Lack of persistence of neutrophils depends on functional pneumolysin**

Previous studies performed in C3H mice demonstrated that pneumolysin expression increases the recruitment of neutrophils following initial colonization (7). In the current study in BALB/c mice challenged with defined, unmarked mutants, we did not observe a pneumolysin-dependent effect on neutrophil recruitment at day 1 postinoculation. Additionally, all strains induced increased levels of the neutrophil-attracting chemokine MIP-2 in nasal lavages,
although MIP-2 levels for each group were highly variable at day 1 postinoculation (data not shown).

Because recruitment in our system did not appear to be pneumolysin dependent, we next examined the same groups for differences in the persistence of the neutrophil response. The number of neutrophils was initially assessed in H&E-stained nasopharyngeal tissue sections at 3 days postinoculation, the time point when the presence of Ag in the NALT was at its maximum. As expected, mice challenged with the wt strain showed neutrophils in the nasal spaces, and RB6-8C5 treatment blocked this response (Fig. 3, A and B). Both the ply− and Ply-W433F mutants lacking functional pneumolysin, however, showed increased numbers of neutrophils (Fig. 3, C and D). We confirmed this observation by quantifying the numbers of neutrophils in nasal lavages by flow cytometry. Cells were labeled with the neutrophil marker Ly-6G and the activation marker CD11b (Fig. 3F–I). Mutants lacking functional pneumolysin had a greater proportion of dual-staining Ly-6G+/CD11b+ cells compared with the wt strain or RB6-8C5-treated control (Fig. 3E). These results suggested that at 3 days postinoculation, the dominant effect of pneumolysin was a diminished persistence of neutrophils.

To examine the effect of pneumolysin on neutrophil survival, lysis was measured with a LDH release assay on ex vivo mouse neutrophils. Treatment with Ply at a dose of ≥0.1 μg/ml resulted in increased cytotoxicity and was significantly more potent than the same concentration of PdB, which is deficient in pore formation due to the W433F mutation (Fig. 4). The expression of purified toxin/toxoid correlated with the ability of live bacteria (10⁷ CFU/ml) to lyse neutrophils in a manner dependent on pneumolysin expression and pore formation (Fig. 4B).

**Pneumolysin-mediated effects on Ag release**

Next, we sought to determine how these interactions between neutrophils and pneumolysin affect Ag delivery. IF staining of nasopharyngeal tissue sections showed the close association between neutrophils and bacteria (Fig. 5A). For the wt strain, staining for type 23F capsular polysaccharide revealed amorphous bacterial Ag that was distinct from intact bacteria, which had the characteristic appearance of diplococci (Fig. 5A). In contrast to the wt strain, the presence of released bacterial Ag within collections of neutrophils was not observed following inoculation of ply− or Ply-W433F mutants (Fig. 5, B, D, and E). In the case of neutrophil-depleted mice, only intact bacteria were seen and these were located along the epithelial surface (Fig. IC). Our results suggested that pneumolysin contributes to the release of bacterial Ag through lysis of neutrophils associated with pneumococci. The release of Ag from intact bacteria would also require lysis of pneumococci, and ex vivo mouse neutrophils were sufficient to kill the opsonized wt strain (Fig. 4C). The expression of functional pneumolysin did not affect the sensitivity of bacteria to neutrophil-mediated killing (data not shown). Thus, neutrophil-mediated lysis of bacteria together with cytotoxicity resulting from pore formation by pneumolysin could account for the requirement of both neutrophils and pneumolysin for efficient Ag release.

**Delivery of Ag to the NALT**

No intact intra- or extracellular bacteria were detected in the NALT, as determined by either IF or a gentamicin protection assay on dissected NALT tissue (data not shown). This suggested that release of Ag into the lumen of the nasal spaces is likely a requirement for efficient Ag delivery. It has been suggested that M-like cells along the mucosal surface of adjacent lymphoid tissue provide a conduit for NALT entry (14). In accordance with this finding, we detected the presence of released pneumococcal Ag in association with cells in the periphery of the NALT, including cells along the mucosal surface in contact with the nasal lumen (Fig. 6, A and B). These Ag-positive cells costained with the M cell marker UEA-1, suggesting uptake of the contents of the nasal lumen by these cells (Fig. 6C). Additionally, the analysis of nasal lavages by flow cytometry did not reveal populations of dendritic cells or monocytes/macrophages in the lumen that might contribute to Ag migration (data not shown).

**Neutrophil- and pneumolysin-mediated effects on clearance**

If delivery of pneumococcal Ag to the NALT functions to activate immune responses, then the efficiency of Ag deposition should affect the rate at which bacteria are cleared from the host. Neither neutropenia nor pneumolysin deficiency had a significant impact on the density of colonizing bacteria at 3 days postinoculation, the time preceding the development of an effective adaptive immune response (Fig. 7A). We then looked at colonization density at 25 days postinoculation, when most BALB/c mice have been shown to clear infection with wt strain (4). It was not possible to evaluate an unbiased effect of neutrophil depletion due to an approximate

![FIGURE 4.](http://www.jimmunol.org/)

**FIGURE 4.** LDH release assay showing that pneumolysin lyses primary murine neutrophils in vitro. A, Purified pneumolysin W433F toxoid PdB exhibits a reduced ability to lyse neutrophils in comparison to equivalent concentrations (μg/ml) of intact Ply (incubation time of 3 h). B, Bacterial-mediated lysis of neutrophils is significantly reduced for strains ply− or Ply-W433F after 3 (gray) and 6 (black) h of coincubation of neutrophils and bacteria. C, Interactions of bacteria and neutrophils (PMNs) lead to killing of wt pneumococci. ***, p < 0.001 relative to the corresponding wt group (A and B) or the control without PMNs in C.**
50% rate of sepsis and death incurred by RB6-8C5 treatment (Fig. 7B). In contrast to animals challenged with the wt strain, most mice inoculated with either ply' or Ply-W433F remained persistently colonized (Fig. 7C). These data confirmed that elaboration of the toxin promotes more rapid bacterial clearance from mucosal surfaces.

To determine whether the effects of pneumolysin expression on clearance were associated with stimulation of the adaptive immune response, levels of mucosal (IgA) or serum (IgG and IgM) Ab to whole-cell wt bacteria or pneumococcal surface protein A were measured by ELISA. These levels, however, correlated with the density of colonization at 25 days postinoculation rather than with

FIGURE 5. The presence of both neutrophils and functional pneumolysin induces the generation of released pneumococcal capsular Ag. At 3 days postinoculation, IF labeling of the nasal lumen and turbinates with anti-Ly-6G (green) and type 23F-specific pneumococcal capsular polysaccharide (red) shows the association of neutrophils with both released bacterial fragments (A, open arrow) and intact pneumococci (A, closed arrow). Fields depicted at higher magnification are shown in A1 and A2, respectively. Released pneumococcal fragments are seen in neutrophil-sufficient mice that are infected with the wt strain (B) but not neutrophil-deficient mice infected with wt (C) or neutrophil-sufficient mice infected with ply' (D) or Ply-W433F (E). Nuclei are labeled by DAPI (blue). Dash represents 10 μm.

FIGURE 6. Delivery of pneumococcal Ag to the NALT. A, IF showing released type 23F-specific capsular polysaccharide Ag (red) associated with cells in the periphery of the NALT and in cells adjacent to the mucosal surface of the NALT (arrows). B, Released type 4-specific capsular polysaccharide Ag (red) associated with cells adjacent to the mucosal surface of the NALT (arrow) 2 days postinoculation with strain TIGR4. C, Released 23F-specific Ag (red) costains (yellow) with the M cell marker UEA-1 (green). Nuclei are labeled by DAPI (blue). Dash represents 10 μm.
and neutrophils promoted delivery of bacterial products to the NALT. The decrease in Ag deposition observed under either neutrophil- or pneumolysin-deficient conditions correlated with prolonged nasopharyngeal colonization, suggesting that the interaction of neutrophils with pneumolysin facilitated an adaptive immune response leading to bacterial clearance.

From these observations, it follows: Why would the bacterium produce a factor that promotes its own clearance? It has been suggested that pneumolysin enhances colonization in vitro by inducing inflammation and tissue damage, which facilitate adherence to the epithelial surface (33). However, the importance of this function in vivo has been contested, as studies examining nasopharyngeal colonization with various strains have reported opposing results (7, 34, 35). These apparent discrepancies may be explained by the different properties of pneumolysin that may affect clearance and may be relevant in some experimental systems while having little effect in others. For example, distinct domains of pneumolysin are known to be responsible for activating the complement pathway and triggering inflammatory responses and apoptosis through direct interaction with TLR4. Disruption of these activities has been shown to affect pneumococcal clearance and the incidence of invasive infection in multiple models (36–40). If either of these properties promoted Ag delivery or clearance in our system, then we should not have observed a deficit due to the Ply-W433F mutation, which does not affect complement activation or TLR4 signaling. Instead, we observed diminished Ag delivery to the NALT and mucosal clearance for the Ply-W433F mutant, suggesting a lesser role for properties of pneumolysin other than its ability to form pores in host membranes.

The ability of pneumolysin to aggregate into pores has also been suggested to promote virulence of the pathogen (41, 42). Recently, however, a study by Kirkham et al. called this idea into question, as more than half of the type 1 strains isolated from patients with invasive pneumococcal disease were shown to contain mutations in the pneumolysin gene, rendering the bacteria unable to form pores (43). Based on the results of our study, it is not surprising that natural isolates that do not form pores exist because these, like the Ply-W433F strain, may benefit from more prolonged colonization. Taken together, these data indicate that pore formation is not necessary for survival of S. pneumoniae and suggest that the almost universal conservation of pneumolysin throughout the >90 pneumococcal serotypes is likely due to an alternative function. We suggest that the inflammatory response to pneumolysin may facilitate host-to-host transmission of the bacterium during early colonization. More efficient transmission may in turn compensate for the shorter duration of carriage caused by enhanced Ag delivery followed by more accelerated adaptive immunity. In this regard, many bacterial toxins expressed by successful mucosal pathogens, such as Bordetella pertussis and Vibrio cholerae, are known to increase secretions that enhance transmission. A further benefit may come from the induction of an inflammatory response to which the organism is resistant because of its detrimental effect on competing members of the local flora (31, 44).

The recruitment and activity of neutrophils may provide additional proinflammatory stimuli. In our studies, we found neutrophils to be effective at promoting pneumococcal degradation, although these cells were ultimately ineffective at controlling early colonization. The killing of pneumococci by neutrophils likely enabled the generation and release of pneumococcal-specific Ag, a process that may be amplified by the bacterium itself because pneumolysin, a cytoplasmic protein, would be released upon cellular breakdown (45). Pneumolysin monomers could then aggregate and form pores capable of causing neutrophil lysis, promoting further Ag release into the nasal spaces. Our studies using whole

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

**FIGURE 7.** Pneumolysin increases clearance of pneumococcal colonization. A. At 3 days postinoculation (10^3 CFU), the presence of neither pneumolysin nor neutrophils affects the colonization density of S. pneumoniae as assessed by quantitative culture of upper respiratory tract lavage. B, Kaplan-Meier survival plot showing the effect of pneumolysin deficiency or treatment with RB6-SC5 to deplete neutrophils on infection with the wt strain. C, At 25 days postinoculation, both ply^−^ and Ply-W433F remain persistently colonized while most of the mice inoculated with the wt parental strain are no longer colonized. Solid line represents median density of colonizing bacteria; dashed line represents the lower limit of detection. For all three panels, ● represents RB6-SC5-treated mice administered wt; ○, neutrophil-sufficient mice administered wt; ▲, neutrophil-sufficient mice administered ply^−^; ▼, neutrophil-sufficient mice administered Ply-W433F; *** p < 0.001 compared with neutrophil-sufficient mice (B) or the wt parent strain (C).

**Discussion**

In this study, we investigated the correlation between the innate immune response to nasopharyngeal infection and the delivery of pneumococcal-specific Ag to the NALT. Capsular polysaccharide was used as a marker of pneumococcal products and Ags because the major T cell-dependent Ag(s) that contribute to cellular immunity have yet to be systematically characterized. Moreover, specific labeling with Abs directed against the surface capsule polysaccharide enabled us to follow the distribution of both intact bacteria and released Ag. By tracking the capsular polysaccharide Ag, we were able to show that the presence of both pneumolysin clearance (data not shown). These findings were consistent with the previously described requirement for cellular rather than humoral immunity upon primary pneumococcal infection (4, 7, 8).

The ability of pneumolysin to aggregate into pores has also been suggested to promote virulence of the pathogen (41, 42). Recently, however, a study by Kirkham et al. called this idea into question, as more than half of the type 1 strains isolated from patients with invasive pneumococcal disease were shown to contain mutations in the pneumolysin gene, rendering the bacteria unable to form pores (43). Based on the results of our study, it is not surprising that natural isolates that do not form pores exist because these, like the Ply-W433F strain, may benefit from more prolonged colonization. Taken together, these data indicate that pore formation is not necessary for survival of S. pneumoniae and suggest that the almost universal conservation of pneumolysin throughout the >90 pneumococcal serotypes is likely due to an alternative function. We suggest that the inflammatory response to pneumolysin may facilitate host-to-host transmission of the bacterium during early colonization. More efficient transmission may in turn compensate for the shorter duration of carriage caused by enhanced Ag delivery followed by more accelerated adaptive immunity. In this regard, many bacterial toxins expressed by successful mucosal pathogens, such as Bordetella pertussis and Vibrio cholerae, are known to increase secretions that enhance transmission. A further benefit may come from the induction of an inflammatory response to which the organism is resistant because of its detrimental effect on competing members of the local flora (31, 44).

The recruitment and activity of neutrophils may provide additional proinflammatory stimuli. In our studies, we found neutrophils to be effective at promoting pneumococcal degradation, although these cells were ultimately ineffective at controlling early colonization. The killing of pneumococci by neutrophils likely enabled the generation and release of pneumococcal-specific Ag, a process that may be amplified by the bacterium itself because pneumolysin, a cytoplasmic protein, would be released upon cellular breakdown (45). Pneumolysin monomers could then aggregate and form pores capable of causing neutrophil lysis, promoting further Ag release into the nasal spaces. Our studies using whole
bacteria, furthermore, add a priori report showing that purified pneumolysin lyses human neutrophils (46).

Our study also addressed how Ag is delivered to inductive sites for the adaptive immune system. Ag released through the interactions of neutrophils and toxin was found in the central regions of the NALT, which demonstrates its ability to sample contents of the lumen of the nasal spaces. We were unable to show direct invasion of the NALT through M cells by live pneumococcus as described by Park et al. for group A streptococcus (14). This could be due to differences in tissue tropism and virulence determinants among these two streptococcal pathogens. There is little prior documentation of pneumococcal interaction with the NALT or its role in the immune response to S. pneumoniae, although a study by Borghesi et al. demonstrated that stimulation of the gut epithelium by introduction of live pneumococci resulted in bacterial internalization (47). Internalization correlated with a morphological increase in gut M cell area, lymphocyte infiltration, and, as shown in a subsequent study by the same group, an up-regulation in the ability of M cells to transport Ags (48).

Although the pneumococcus is not among the flora naturally present in the gut, the ability of M cells in the mucosa to transport the pathogen through the epithelium suggests the possibility that similar sampling might occur in the respiratory mucosa as well. It is likely, however, that this is relevant only to released pneumococcal Ag, because we were unable to detect intact pneumococci within the NALT and to show colocalization of released pneumococcal Ag with M cells.

We cannot exclude the possibility that other cells might be involved in transporting pneumococcal Ag from the nasal spaces to the NALT. It is possible that dendritic cells or professional phagocytes, which have been shown to internalize pneumococci and pneumococcal polysaccharide, are recruited to the site of infection (49, 50). There, they may interact with recruited T cells, which then travel to the NALT, or the phagocytes may undergo migration themselves. Alternatively, resident phagocytic cells, which interdigitate between the epithelium, may sample the luminal space much in the same manner as M cells. IF and flow cytometry, however, did not indicate the recruitment of cells other than neutrophils into the nasal spaces in response to colonization. This observation also makes it unlikely that results based on RB6-BC5 treatments to deplete neutrophils could be explained by its previously described effects on other subpopulations of Ly-6G-expressing monocytes, macrophages, or dendritic cells (51).

In summary, our findings offer a paradigm for the process of Ag delivery to immune inductive sites in the upper respiratory tract airway during colonization. The presence of free Ag in the lumen appeared to permit sampling by the NALT in a manner consistent with uptake by M cells along the adjacent mucosal surface. Ag delivery, moreover, was facilitated by host and microbial factors that released microbial products from intact organisms. In the case of S. pneumoniae, this involved bacterial degradation by neutrophils in conjunction with neutrophil lysis caused by the pore-forming bacterial toxin, pneumolysin. The contribution of neutrophils explains why peak Ag delivery to the NALT coincided with the acute inflammatory response in the nasal spaces. In this regard, this study also provides a demonstration of how the characteristics of the acute inflammatory response affect subsequent immunity leading to mucosal clearance.

Disclosures

The authors have no financial conflicts of interest.

References


