Bacterial exploitation of phosphorylcholine mimicry suppresses inflammation to promote airway infection

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Introduction
Neutrophils comprise an essential component of the acute inflammatory response to pathogens, particularly that against extracellular bacteria that reside on mucosal surfaces (1, 2). Typically first among leukocytes recruited to sites of infection, neutrophils exert potent bactericidal activity, impede microbial dissemination from epithelial barriers, and inhibit bacterial transmission between hosts (3–5). In turn, opportunistic microbes have evolved strategies to evade the neutrophils they elicit, the mechanisms of which have long been the subject of intensive study (6, 7). However, as the understanding of neutrophil biology has advanced, it has become clear that neutrophil bactericidal capacity is regulated dynamically and locally at inflamed sites (8) and that some pathogens directly manipulate the phagocyte activation state to inhibit microbial clearance at sites of infection (9, 10). Our understanding of the mechanisms by which neutrophil phagocytic function is suppressed in vivo remains incomplete.

Streptococcus pneumoniae, known as the pneumococcus, was among the first pathogens for which neutrophil evasion mechanisms were proposed (11–13). A leading cause of gram-positive bacterial pneumonia, sepsis, and meningitis, the pneumococcus has been long known to be targets for immune recognition (26). Together, these studies have observed that progression from benign carriage to invasive infection often occurs only a few days after acquisition of upper airway infection (16–18). This coincides with the peak of neutrophil influx into the airway lumen and implies that pneumococcus-neutrophil interactions in the upper airway may govern the balance between bacterial clearance and disease (19, 20).

Pneumococcal resistance against neutrophils has traditionally been ascribed to its thick polysaccharide capsule, which serves to shield the bacterium from opsonization with complement or antibodies that would otherwise hasten phagocytic uptake (21, 22). However, in contrast to that in the lungs or bloodstream (23), pneumococcal resistance to neutrophils in the upper airway does not rely on antibody or complement evasion (24, 25). Further, colonizing pneumococci express markedly less capsular polysaccharide compared with blood and lung isolates, exposing cell wall components long known to be targets for immune recognition (26). Together, this suggests that capsule-mediated antiphagocytosis fails to fully explain pneumococcal neutrophil evasion in the upper airway. We sought to determine whether pneumococci instead disarm the phagocytic function of neutrophils recruited to the airway lumen, rendering them unable to mediate acute bacterial clearance.

Molecular mimicry is among the most widely conserved mechanisms by which bacteria evade immunity, exploiting the host’s inability to recognize self-derived molecular structures (27). The pneumococcus, like many other airway bacteria, capitalizes on this vulnerability by displaying the host-derived small molecule phosphorylcholine (ChoP) on its surface. Decoration of pneumococcal cell wall components with ChoP is essential for bacterial fitness and is necessary to inhibit bacterial opsoniza-
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Figure 1. Pce-deficient pneumococci exhibit impaired persistence in the upper airway and elicit the recruitment of more activated, viable, and durable neutrophils to the nasal lumen. (A) Bacterial clearance in mice inoculated with WT pneumococci, strain P1121 (Type 23F), with (white circles) or without (black circles) systemic neutrophil depletion (n = 4–5 mice per condition, limit of detection [LOD] = 2). (B) Survival of WT P1121 (black) or P1121Δpce (gray) pneumococci in the murine upper airway (n = 4–14). (C) Day-7 survival of P1121Δpce mutant generated by in-frame, unmarked deletion (Δpce) and with genetic correction (Δpce::pce) (n = 5). (D) Day 7 survival of WT and Δpce pneumococci on a type 4 (T4, TIGR4) pneumococcal genetic background (n = 5). (E) Quantification of neutrophils (CD45+CD11b+Ly6G) obtained from the upper airway lumen by nasal lavage before (n = 3) and after (n = 4–11) inoculation with WT (black) or Δpce (gray) pneumococci. (F) Flow cytometric characterization of luminal neutrophils elicited by infection with WT or Δpce pneumococci on day 4 p.i. (n = 6–8). Note that not all axes are continuous, and gaps in axes represent gaps in time. Dotted lines represent the LOD. Statistical significance was assessed by 1-way ANOVA with Newman-Keuls post test for comparisons of more than 2 conditions (A, B, and E). Student’s t test for 2-group comparisons (C and D), and 1-sample Student’s t test relative to null = 1 for relative MFI measurements (F). *P < 0.05, ***P < 0.001.
inoculated with WT or Pce-deficient (Δpce) pneumococci were sacrificed and lavaged for bacterial enumeration 1, 3, 4, and 7 days p.i. (Figure 1A). These results suggested that, despite their rapid influx into the airway lumen following acquisition of infection (43), neutrophils failed to exert significant bactericidal pressure against S. pneumoniae in the upper airway.

Pneumococcal Pce esterase inhibits bacterial clearance and neutrophil activation. We next asked whether Pce esterase contributes to pneumococcal evasion of acute clearance from the airway. Mice inoculated with WT or Pce-deficient (Δpce) pneumococci were sacrificed and lavaged for bacterial enumeration 1, 3, 4, and 7 days p.i., corresponding to the period during which neutrophil inflammation is most prominent (20). While WT bacteria persisted at maximal density over the first week of infection, Δpce pneumococci exhibited a survival defect that exceeded 20-fold by day 7 p.i. (Figure 1B). Notably, WT and Δpce bacterial loads were equivalent at 1 day p.i., suggesting that differential inoculum retention or impaired establishment of infection was unlikely to explain the mutant’s subsequent persistence defect. The poor survival of Δpce pneumococci was recapitulated with an independently constructed unmarked, in-frame deletion mutant, restored upon genetic correction (Figure 1C) and preserved in a distinct serotype (type 6A) pneumococci of invasive strain P1547 (type 6A) (n = 12 mice per condition from 3 independent experiments). Statistical significance was assessed by the Mantel-Cox test. (B) Enumeration of CFU in the blood of mice infected with WT or Δpce P1547 pneumococci as above (n = 5; LOD = 14). (C) Infant murine transmission. Upper airway lavage CFU enumerated from index (white circles, n = 3–4) and contact (black circles, n = 14–18) pups on day 14 of life and after index mice were inoculated with WT (black) or Δpce (gray) pneumococci on day 4 of life. All pups were infected i.n. with influenza on day 8. Numerical values above contact mice columns represent the percentage of acquisition (LOD = 10). Transmission data reflect 3 independent experiments. In B and C, statistical significance was assessed by 1-way ANOVA with Newman-Keuls post test. *P < 0.05, **P < 0.01, ***P < 0.001.

We then performed flow cytometry to characterize the acute inflammatory response to WT and Δpce bacteria. Neutrophils dominated the cellular infiltrates elicited by both WT and Δpce pneumococci (>95% of CD45+ events, Supplemental Figure 1C). Despite lower bacterial density in the airway, mice inoculated with the Δpce mutant exhibited elevations in the maximum density and, more markedly, the duration of neutrophil influx (Figure 1E). Congruent with bacterial load measurements, neutrophils persisted in the airway lumen through day 14 p.i. in Δpce-infected mice but were nearly absent in response to WT pneumococci (Supplemental Figure 1B).

The more robust acute inflammatory response observed in the absence of Pce led us to hypothesize that the enzyme may perturb the qualitative capacity of neutrophils to persist and clear bacteria. Accordingly, we predicted that neutrophils recruited by Δpce pneumococci would exhibit greater activation and superior phagocytic capabilities. To assess antimicrobial capacity in vivo, we functionally characterized the neutrophils responding to WT versus Δpce bacteria by flow cytometry (Figure 1F). Compared with neutrophils elicited by WT pneumococci, those elicited by the mutant exhibited significantly elevated expression of CD11b (CR3) and CD64 (FcγRI) — which serve as both activation markers and bacterial uptake receptors (2, 39, 44) — and greater than 2-fold enhanced shedding of CD62L (L-selectin), a marker for inflammation-induced neutrophil activation (45, 46). Luminal neutrophils elicited by Δpce bacteria included approximately twice as many viable cells compared with those responding to WT, a finding consistent with their enhanced persistence in the airway lumen. Last, Δpce-recruited neutrophils exhibited an approximately 5-fold increase in ROS production per cell (as detected by CM-H₂DCFDA dye staining) over those recruited by WT bacteria, underscoring their enhanced bactericidal capacity (3). Together, these findings implied that Pce esterase impairs the cellular viability and phagocytic functionality of neutrophils upon their arrival in the airway lumen.

Pce promotes invasive pneumococcal disease and bacterial transmission between mice. A number of clinical and animal studies have found pneumococcal load in the upper airway to be strongly associated with both the onset and severity of subsequent invasive disease (16, 47) as well as the risk of pneumococcal transmission between hosts (5, 18). We asked whether the survival deficit exhibited by Δpce pneumococci during infection of the upper airway corresponded with differences in these clinically important outcomes. To gauge relative invasive disease risk, we inoculated mice i.n. with
WT or Δpce pneumococci generated from a mouse-invasive strain (serotype 6A) and tracked survival over 9 days (Figure 2A). While approximately 60% of mice infected with WT bacteria were moribund within 5 days from pneumococcal sepsis, mice inoculated with the mutant were significantly protected, suffering less than 10% lethality over the course of the experiment. This difference in survival corresponded with substantial attenuation in bacterial invasion of the bloodstream among Δpce pneumococci (Figure 2B).

Δpce pneumococci were similarly impaired in an infant mouse model of pneumococcal transmission (5, 48). Within litters of mice, we inoculated 1–2 “index” pups with either WT or Δpce bacteria and quantified the acquisition and load of pneumococci among previously uninfected “contact” pups. In line with previous findings (48), litters exposed to WT bacteria exhibited 64% transmission; however, only 6% of control mice from litters exposed to Δpce pneumococci acquired bacteria (Figure 2C). This corresponded with greater than 300-fold lower bacterial loads among index mice infected with the mutant. Taken together, these data provide evidence that the poor survival of Δpce pneumococci in the upper airway crosses a key threshold under which disease and transmission are nearly abrogated.

Pce prevents accumulation of PAF in the upper airway lumen. Next, we analyzed the mechanism by which Pce esterase impairs neutrophil function in the upper airway. Studies using purified, recombinant enzyme have shown that Pce is capable of hydrolyzing a wide range of molecules bearing ChoP in vitro, including PAF (ref. 49 and Figure 3A, diagram). To directly quantify the impact of Pce on airway luminal PAF levels, we inoculated mice with PBS (mock) or WT or Δpce pneumococci and pooled lavages from 5 mice per group on day 3 p.i. After lipid extraction and liquid chromatography (LC) to purify PAF, we subjected samples from each condition to high-resolution electrospray ionization/mass spectrometry (ESI/MS). Lavage fluid from mice inoculated with Δpce bacteria harbored PAF at a concentration of approximately 3 nM, while mock- and WT-infected mice secreted no PAF detectable by LC-ESI/MS (Figure 3A), demonstrating that Pce prohibits the accumulation of PAF in the upper airway lumen during pneumococcal infection.

We also examined the expression of genes essential for PAF signaling in the airway epithelium, as PAF is known to stimulate the transcription of its own synthetic enzymes and receptor via a positive feedback loop (50, 51). At 3 days p.i. with WT or Δpce bacteria, we obtained lavages with tissue lysis buffer and quantified mucosal transcripts by quantitative RT-PCR (qRT-PCR) in each condition relative to mock infection. Transcript levels of the PAF synthetic enzyme lyso-PAF acetyltransferase (Lpcat2) and the PAF receptor (Ptafr) were markedly elevated among mice infected with Δpce pneumococci (Figure 3B). In contrast, transcription of neutrophil chemokines Cxcl1 and Cxcl2 trended lower in mice inoculated with Δpce pneumococci (n = 6–10 mice per condition). All transcripts were normalized to GAPDH controls and are displayed relative to mice mock-infected with PBS (dotted lines). *P < 0.05 by Student’s t test.
Our data show that Pce esterase hydrolyzes ChoP and abrogates PAF-mediated stimulation of neutrophils, local PAFR antagonist restored survival of Δpce pneumococci to levels comparable to those of WT pneumococci (Figure 4C). PCA-4248 had no direct effect on pneumococcal survival or growth in vitro (Supplemental Figure 2B). Importantly, no additional increase in the density of Δpce bacteria was seen upon PCA-4248 treatment of neutrophil-depleted mice, underscoring that the predominant impact of elevated airway PAF in the absence of Pce is stimulation of neutrophil function. Last, we found that bacterial loads on day 7 p.i. were equivalent among WT and Δpce pneumococci in Ptafr−/− mice and their littermate controls on day 7 p.i. (*P < 0.05, **P < 0.01, and ***P < 0.001 by 1-way ANOVA with Newman-Keuls post test).

To assess whether local PAF signaling contributed to Pce-mediated neutrophil evasion, we performed the same 7-day infection with daily i.n. administration of PCA-4248, a selective antagonist of PAF receptor (PAFR) (52–54). Similar to our findings upon depletion of neutrophils, local PAFR antagonism restored survival of Δpce pneumococci to levels comparable to those of WT pneumococci (Figure 4C). PCA-4248 had no direct effect on pneumococcal survival or growth in vitro (Supplemental Figure 2B). Importantly, no additional increase in the density of Δpce bacteria was seen upon PCA-4248 treatment of neutrophil-depleted mice, underscoring that the predominant impact of elevated airway PAF in the absence of Pce is stimulation of neutrophil function. Last, we found that bacterial loads on day 7 p.i. were equivalent among WT and Δpce pneumococci in Ptafr−/− mice and their littermate controls on day 7 p.i. (*P < 0.05, **P < 0.01, and ***P < 0.001 by 1-way ANOVA with Newman-Keuls post test).

Neutrophil depletion or inhibition of PAF signaling renders Pce dispensable for S. pneumoniae persistence. Our data show that S. pneumoniae lacking Pce esterase exhibit a survival defect during acute infection of the airway, which corresponds with elevated levels of PAF and enhanced activation of luminal neutrophils. We next asked whether neutrophils and PAF signaling play a causal role in driving the mutant’s rapid clearance. To determine whether the poor survival of Δpce bacteria could be rescued in the absence of infiltrating neutrophils, mice were treated with neutrophil-depleting anti-Ly6G or IgG2a isotype control antibodies on days −1, +1, and +4 after inoculation with WT or Δpce pneumococci. Effective neutrophil depletion was verified in blood and nasal lavage fluid by flow cytometry (Figure 4A), and bacterial CFU from neutropenic and control mice were enumerated from nasal lavages on day 7 p.i. While the Δpce mutant retained an approximately 20-fold survival defect in isotype control–treated animals, its survival was restored to WT levels in neutrophil-depleted mice (Figure 4B). This confirmed that neutrophils were required for the enhanced clearance of the mutant and that Pce esterase functions to impair neutrophil-mediated bactericidal function.

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significantly different in terms of growth characteristics in vitro (Supplemental Figure 2A), sensitivity to complement deposition (Supplemental Figure 3A), capsule expression levels (Supplemental Figure 3B), or cell surface ChoP accessibility (Supplemental Figure 3C) as assessed by bacterial flow cytometry.

Using recombinant Pce esterase (rPce), we next confirmed that the enzyme bears efficient ChoP hydrolysis activity upon incubation with chromogenic substrate p-nitrophenylphosphorylcholine (pNPPC), which yields p-nitrophenol upon removal of its ChoP moiety (Figure 5C) (56). To assess whether rPce directly inhibits PAF-mediated stimulation of neutrophil activation and function in vitro, we pretreated PAF at a range of physiologic concentrations centered around 3 nM (the concentration detected by ESI/MS) with rPce enzyme or PBS control. We applied the conditioned PAF media to the phagocytes and quantified upregulation of CD11b and CD64 receptor expression on murine neutrophils by flow cytometry (Figure 5D).

Figure 5. Pce esterase hydrolyzes ChoP from conjugated substrates and directly inhibits PAF-mediated stimulation of neutrophil activation and function in vitro. Killing of WT (black) or Δpce (gray) P1121 pneumococci in vitro by murine (A) or human (B) neutrophils at the indicated neutrophil/bacterium ratios, after preopsonization with BRS. Bacterial survival was measured relative to control assays in the absence of neutrophils (dotted line). HI, heat inactivated. (C) Kinetic time course of p-nitrophenol liberation (absorbance at 415 nm) after incubation of pNPPC with recombinant Pce enzyme (rPce, black line). Assays were repeated in the absence of Pce enzyme or pNPPC substrate or in the presence of 250 mM EDTA. $K_{\text{cat}}$, observed kinetic rate constant. (D) MFI quantification of bacterial uptake receptors CD11b and CD64 on murine neutrophils treated with PAF that was preincubated with rPce (black) or PBS (white). PAF stimulation assays were repeated with 10^{-5} M of the PAFR antagonist PCA-4248 as a specificity control. Killing assays using WT pneumococci (PMN/bacterium ratio, 1,000:1) were performed after PAF-mediated murine (D) or human (E) neutrophil stimulation in the presence (black) or absence (white) of rPce. Top dotted line denotes 100% bacterial survival; bottom dotted line denotes average survival in the absence of PAF. For all panels, data averages reflect at least 3 independent experiments (3–4 independent biological replicates for cellular assays). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ by Student’s $t$ test for all pairwise comparisons.
While increasing PAF concentrations correlated with upregulation of both receptors under the PBS control condition, no such correlation was seen if PAF was preincubated with rPce. Similarly, pretreatment of PAF with rPce abrogated the increased killing capacity conferred when PAF was applied to murine or human neutrophils (Figure 5, D and E). Collectively, these findings reveal that Pce functions to hydrolyze ChoP from conjugated substrates, and its processing of PAF directly inhibits neutrophil activation and phagocyte function.

**Exogenous stimulation of neutrophils in situ overwhelms Pce-mediated immune evasion.** Having established that Pce inhibits acute clearance of *S. pneumoniae* in vivo by depriving neutrophils of an essential stimulatory ligand, we posited that this immune evasion mechanism could be overcome if luminal neutrophils were sufficiently activated. We tested this by performing 7-day WT and Δpce pneumococcal infection experiments with daily i.n. treatments with an excess of either PAF or fMLP, a bacterial peptide that stimulates neutrophil chemotaxis and activation through pathways distinct from those of PAF (39, 57). At day 7 p.i., mice inoculated with WT pneumococci and treated with PAF (Figure 6A) or fMLP (Figure 6D) exhibited significantly enhanced clearance, such that WT bacterial loads resembled that of Δpce, while the mutant’s survival was not substantially affected upon stimulant treatment. PAF and fMLP exerted no detectable effect on pneumococcal growth or viability in vitro (Supplemental Figure 2, C and D).

Critical, by recruiting these treatments after systemic neutrophil depletion restored survival of both pneumococcal strains to levels seen for WT bacteria in mice treated with vehicle controls (Figure 6, A and D), reinforcing that neutrophils were responsible for the observed increase in clearance. Enhancement of WT pneumococcal clearance upon PAF treatment corresponded with an increase in the number of neutrophils in the airway lumen to levels similar to those seen during Δpce infection. In contrast, neutrophil numbers elicited by Δpce pneumococci were unaffected by PAF administration (Figure 6B). These results suggested that introduction of excess PAF ligand overwhelmed the capability of Pce esterase to mediate immune evasion for WT bacteria, yielding neutrophil phenotypes and bacterial survival that mimicked infection in the absence of Pce. Consistent with its known role as a strong chemotactic ligand (58), fMLP treatment stimulated a substantial elevation in airway neutrophil recruitment, but neutrophil numbers were again equalized among WT- and Δpce-infected mice (Figure 6E). Last, flow cytometric analysis of luminal neutrophils revealed that treatment with PAF or fMLP enhanced the relative expression of CD11b and CD64 on cells elicited by WT pneumococci to levels seen in those recruited by Δpce, abrogating the relative difference in activation observed in the absence of stimulation in situ (Figure 6, C and F). Taken together, these results serve to reinforce that Pce promotes pneumococcal persistence through fine regulation of neutrophil activation and that clearance of WT bacteria can be substantially accelerated by stimulating neutrophil function.

**Subversion of PAF signaling is conserved in Haemophilus influenzae, another ChoP-expressing pathogen of the airway.** We sought to determine whether exploitation of ChoP mimicry to suppress PAF represents a conserved mechanism among other ChoP-expressing bacterial pathogens. We focused on *Haemophilus influenzae*, another ChoP-expressing pathogen of the upper respiratory tract.
as it displays ChoP on surface-exposed lipooligosaccharide chains (28), remains an important cause of respiratory disease worldwide (59, 60), and could be investigated using a murine model of upper respiratory tract infection (61). No direct homologs for pneumococcal Pce were apparent in H. influenzae by sequence analysis. However, previous work from our laboratory suggested that the highly conserved, surface-bound phosphodiesterase lipoprotein GlpQ (also known as protein D) bears the ability to efficiently hydrolyze ChoP from conjugated substrates (62). Previously ascribed a role in bacterial acquisition of choline from host cells, GlpQ has long been known to be important for virulence during mucosal infection, though the nature of its contribution has remained incompletely understood (63). We hypothesized that this ChoP-binding enzyme functioned analogously to pneumococcal Pce esterase and contributed to PAF evasion during airway infection.

To confirm previous reports that GlpQ contributes to hydrolysis of ChoP (62), we applied WT and ΔglpQ H. influenzae to chromogenic pNPPC assays and measured absorbance after 120 minutes compared with that generated by WT and Δpce pneumococci (Figure 7, A and B). Note that GlpQ cleaves ChoP at the phosphoester bond proximal to choline and therefore requires the addition of exogenous alkaline phosphatase enzyme to reactions for chromogenic activity in this assay (Figure 7A, arrows). Similar to the results for Δpce pneumococci, ChoP hydrolysis activity by H. influenzae was significantly impaired in the absence of GlpQ, though some residual activity remained (Figure 7B). These results confirmed that native GlpQ hydrolyzes ChoP efficiently.

We next asked whether GlpQ contributes to bacterial persistence in murine upper airway infection. Mice inoculated with WT or ΔglpQ H. influenzae were sacrificed on days 1 and 2 p.i. for bacterial enumeration. By day 2, ΔglpQ bacteria exhibited a survival defect exceeding 20-fold (Figure 7C). Flow cytometric analyses of lavage fluid suggested enhanced influx of neutrophils during acute infection with the ΔglpQ mutant compared with that observed for WT bacteria (Figure 7D), and neutrophils elicited by the mutant exhibited significantly elevated bacterial uptake receptor expression by day 2 p.i. (Figure 7E). To establish whether neutrophils drive ΔglpQ persistence defects, we repeated 2-day infections after anti-Ly6G or IgG2a isotype antibody treatments on days –1 and +1 after inoculation. Akin to results observed with Δpce pneumococci, neutropenia rescued ΔglpQ survival such that it resembled that of WT H. influenzae (Figure 7F). ΔglpQ survival was also rescued...
in Ptafr\(^{-/-}\) mice, while littermate control mice recapitulated the mutant defect seen previously, indicating that intact PAF signaling is essential for enhanced bacterial clearance in the absence of GlpQ (Figure 7G). Importantly, neutropenia did not lead to significantly enhanced bacterial survival in Ptafr\(^{-/-}\) mice, suggesting that PAF stimulation plays an important role in regulating neutrophil function during H. influenzae infection of the airway. Collectively, these findings reveal that GlpQ is critical for limiting neutrophil responses in vivo through inhibition of PAF signaling.

Last, we performed neutrophil bactericidal assays in vitro to assess whether preincubation of PAF with H. influenzae bearing GlpQ hindered its ability to stimulate neutrophil-mediated phagocytic function when compared with preincubation with \(\Delta glpQ\) bacteria. We mixed neutrophils pretreated with each enzyme-conditioned PAF solution with opsonized WT bacteria. We focused our investigations entirely on PAF C16 (1-O-hexadecyl-2-O-acyl-sn-glyceryl-3-phosphorylcholine), the most abundant and well-characterized member of a diverse set of PAF-like lipids secreted during inflammation. While some PAF-like lipids can stimulate neutrophil bactericidal capacity, nearly all bind most avidly to receptors other than PAFR (70). A number of studies have shown that even small modifications to the molecular structure of PAF significantly disrupt its binding and signaling potency through its cognate receptor (71). Since we found that the enhanced neutrophil activation observed during infection with \(\Delta pce\) pneumococci was abrogated entirely upon PAFR blockade in vivo or when using PAFR-deficient mice, we concentrated our analyses on PAF. Whether other PAF-like lipids are regulated similarly by Pce during pneumococcal infection remains a subject of ongoing investigation.

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Our work demonstrated that Pce-mediated PAF degradation results in functional impairment of neutrophils responding to pneumococcal infection, rendering them unable to mediate efficient phagocytosis. Recent studies have suggested that, in addition to suppressing clearance, recruiting an ineffective acute inflammatory response can be directly beneficial for bacterial fitness. Increased mucus production can provide nutrients to stimulate bacterial growth at mucosal surfaces (72), inflammatory influxes can enhance bacterial shedding from the nasopharynx to promote transmission between hosts (48), and the recruitment of neutrophils can provide an inflammatory milieu that may neutralize bacterial competitors among the flora (9). These advantages rely on phagocytes that alter the mucosal environment without presenting significant bactericidal pressure. For the pneumococcus, Pce-mediated neutrophil suppression may constitute a central mechanism through which inflammation can be utilized to the advantage of the microbe.

A wide range of extracellular bacteria display ChoP on their surfaces and express enzymes that govern ChoP hydrolysis and turnover, suggesting that other pathogens may harbor unappreciated strategies for evading clearance through mimicry-driven degradation of PAF (28). Along with the pneumococcus, H. influenzae, Neisseria meningitidis, and Neisseria gonorrhoeae carry ChoP as surface modifications, and most of these pathogens use ChoP to bind PAFR directly, reflecting a common strategy of passive, structural mimicry of PAF (73-75). Our work implies that active, enzymatic mechanisms exploiting ChoP mimicry may underlie the well-described resistance of these organisms to neutrophils and may expose a broadly applicable target for therapeutic intervention. To
this end, we investigated neutrophil evasion by \textit{H. influenzae} in the upper airway. Despite bearing no apparent homologs for \textit{pce}, we found that \textit{H. influenzae} harbors an analogous mechanism for mimicry-driven, enzymatic subversion of PAF through ChoP hydrolysis by the surface-bound phosphodiesterase GlpQ. The functional similarity of these structurally unrelated esterases, used by disparate pathogens occupying the same upper airway niche, suggests that PAF-mediated inflammation may have necessitated convergent evolution (27) aimed at manipulating and neutralizing PAF activity to achieve fitness in the airway environment. Our findings also suggest that GlpQ does not govern the entirety of ChoP hydrolytic activity mediated by \textit{H. influenzae}, as some residual enzymatic activity persists among \textit{AglpQ} mutants. The identity and functions of these other contributors remain under investigation.

The central importance of regulating PAF-mediated inflammation is further exemplified by microbes that do not express ChoP but disrupt PAF signaling by means other than molecular mimicry. \textit{Staphylococcus aureus} has been shown to bind leukocytes and platelets and directly modulate PAFR signaling (76, 77). \textit{Streptococcus pyogenes} uses a secreted enzyme to cleave acetyl groups from PAF ligand and inhibit neutrophil chemotaxis during invasive skin infection (78). It remains conceivable that additional mechanisms for PAF disruption by \textit{S. pneumoniae} and \textit{H. influenzae} exist independently of ChoP hydrolysis activity. Together, this emphasizes that microbial manipulation of PAF signaling may be critical for successful immune evasion and that targeting convergent bacterial strategies to this end may be a promising avenue for antimicrobial interventions.

The tight regulation of PAF signaling mediated by the pneumococcus and other extracellular bacteria suggests that these neutrophil evasion mechanisms could be overcome if PAF levels are elevated sufficiently at the site of infection. Accordingly, we found that stimulating neutrophils in situ during pneumococcal infection with excess PAF restored their activation state and phagocytic capacity, resulting in enhanced bacterial clearance. This implies that a threshold concentration of PAF may define the balance between effective bacterial evasion and neutrophil-mediated clearance. While delivering PAF itself is unlikely to be a clinically tractable method to overcome such a threshold, targeting host-encoded negative feedback mechanisms that regulate PAF levels may be more promising. Lipoprotein-associated phospholipase \textit{A} \textsubscript{2}, also known as PAF acetylhydrolase, is secreted by the host, has been shown to mediate PAF hydrolysis, and can dampen PAF-mediated inflammation in vivo (79). It is released into the upper airway lumen during respiratory inflammation and has been shown to be upregulated during pneumococcal infection, and clinical trials have demonstrated that an antagonist, darapladib, is safe in humans (80–82). While studies aimed to assess the effectiveness of targeting PAF acetylhydrolase in combating pneumococcal and other bacterial infections are ongoing, this may serve as an important example of how mechanistic investigations of bacterial immune evasion may reveal rational targets for host-directed antimicrobial therapies.

Methods

\textbf{Bacterial strains.} \textit{S. pneumoniae} strains P1121 (type 23F clinical isolate), TIGR4 (type 4 isolate), and P1547 (a mouse-virulent type 6A isolate) were grown in tryptic soy (TS) broth at 37°C to mid-log phase, as described previously (19, 83, 84). Mutants lacking ChoP esterase (\textit{apce}) were derived for each strain from an insertion-duplication mutant (56) and used for all experiments except where specified. Independently, we created an in-frame, unmarked \textit{apce}-deletion mutant and a genetically corrected revertant (\textit{apce:apce}) by previously described methods (41). Refer to the Supplemental Methods for details and to Supplemental Table 1 for primers used in mutagenesis. Spontaneously streptomycin-resistant isolates of \textit{H. influenzae} Eagan (a type b encapsulated strain) and an isogenic mutant lacking the surface phosphodiesterase GlpQ (\textit{aglpQ}) were used as described previously (62). \textit{H. influenzae} was grown to mid-log phase shaking at 37°C in brain-heart infusion (BHI) broth supplemented with 2% Fildes Enrichment (Thermo Scientific) and 2 μg/ml β-NAD (Sigma-Aldrich) (sBHI).

\textbf{Murine model of upper airway infection.} Six- to eight-week-old C57BL/6 mice were obtained from The Jackson Laboratory. PAFR-deficient (\textit{Ptafr\textsuperscript{−/−}}) mice on a C57BL/6 background (\textit{Ptafr\textsuperscript{−/−}}) were a gift of Elaine Tuomanen (St. Jude Children’s Research Hospital, Memphis, Tennessee, USA) (85, 86). All KO mice were bred from heterozygotes and compared with littermate controls. For upper airway infection with \textit{S. pneumoniae} or \textit{H. influenzae}, mice were inoculated i.n. with 10\textsuperscript{7} CFU of mid-log phase bacteria suspended in 10 μl sterile PBS. To prevent aspiration of the inoculum from the upper airway into the lungs, mice were not anesthetized during inoculation. We obtained upper airway lavages upon sacrifice through tracheal cannulation and expression of 200 μl sterile PBS through the nares. Lavage samples from pneumococcal infections were plated on TS agar supplemented with 5 to 20 μg/ml neomycin and 5,000 U catalase per plate (Worthington Biochemical). To discern insertion-derived and in-frame deletion \textit{apce} mutants from WT pneumococci, plates were supplemented with 1 μg/ml erythromycin or 200 μg/ml streptomycin, respectively. Lavage samples from mice infected with \textit{H. influenzae} were plated on sBHI agar supplemented with 100 μg/ml streptomycin. \textit{AglpQ} mutants were discerned from WT \textit{H. influenzae} by supplementation with 20 μg/ml kanamycin. CFU counts were enumerated by quantitative culture after overnight incubation at 37°C in 5% CO\textsubscript{2} (72).

\textbf{Flow cytometry.} Nasal lavages were pelleted at 1,200 g for 5 minutes and resuspended in PBS containing 1% BSA. FcR blocking was achieved with 1:100 dilution of anti-CD16/32 (clone 2.4G2; BD Biosciences), and cell viability was assessed by staining with Fixable Viability Dye eFluor 780 (eBioscience) according to the manufacturer’s instructions. Neutrophils were immunophenotyped by staining with fluorophore-conjugated antibodies (diluted 1:150) against the following surface markers: CD45 (clone 30-F11; eBioscience); Ly6G (clone 1A8; BioLegend); CD11b (clone M1/70; BioLegend); CD64 (clone X54-5/7; BioLegend); and CD62L (clone MEL-14; BioLegend). Neutrophil ROS were quantified after incubation with 10 μM CM-HDCFDA (Life Technologies) according to the manufacturer’s instructions (87, 88). All cytometry was performed using a BD LSR II flow cytometer and analyzed with FlowJo software.

\textbf{Bacterial transmission model.} Infant mouse pneumococcal transmission experiments were performed as described previously (5, 48). On day 4 of life, we inoculated 1–2 index pups per litter (approximately 1 in 4) with 2,000 CFU of \textit{S. pneumoniae} strain P1121 in 3 μl PBS. On day 8 of life, all pups were infected i.n. with 2 × 10\textsuperscript{4} TCID\textsubscript{50} \textit{influenza A} strain HKx31, as influenza coinfection is required for pneumococcal colonization.
transmission between mice (89). On day 14, all pups were sacrificed, and upper airway bacterial loads were quantified among index and previously uninfected contact mice.

LC-ESI/MS quantification of PAF from the murine upper airway lumen. PAF-C16 was extracted and quantified from murine upper airway lavage fluid pooled from 5 mice per condition on day 3 of infection (performed in triplicate). Briefly, 0.5 ml lavage fluid was spiked with 2 ng deuterated PAF-C16-d4 (Cayman Chemical) as a quantification standard, extracted in methanol with shaking, and resuspended in isopropanol/acetone/2H2O/water (3:5:2; v/v/v). Reverse-phase separations of 3-μl injections were conducted using a nano-AQCUITY UPLC system and XBridge BEH130 C18 column (Waters Corp.) at 1.5 μl/minute. High-resolution LC-ESI/MS quantification was performed using a recently calibrated LTQ XL-Orbitrap hybrid mass spectrometer (Thermo Scientific) in positive ion mode and with a Michrom captive spray ESI source. Data analysis was performed using Xcalibur software (Thermo Scientific) from raw mass spectral data.

qRT-PCR. RNA was extracted from the upper airway mucosa through lavage with 500 μl RLT lysis buffer (QIAGEN) (90). After isolation of total RNA (RNaseasy Kit; QIAGEN), cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and qRT-PCR was performed using 10 ng cDNA, 0.5 μM primers, and SYBR Green Master Mix (Life Technologies). Differential RNA expression was quantified using the ΔΔCt method relative to Gapdh transcript levels. qRT-PCR primer sequences are listed in Supplemental Table 1.

Neutrophil depletion and i.n. drug treatments. Systemic depletion of murine neutrophils was achieved by i.p. injection of 250 μg anti-Ly6G antibody (clone 1A8; Bio X Cell) or IgG2a isotype control antibody (clone 2A3; Bio X Cell) at the indicated time points (Results and Figures 4 and 6). Neutropenia was confirmed by flow cytometry and microscopic inspection using Shandon Quin-Diff Stains (Thermo Scientific). For i.n. drug treatments, 0.1 or 1 μg PAF (Cayman Chemical), PAF receptor antagonist PCA-4248 (Tocris), or fMLP (Sigma-Aldrich) was instilled daily (days 1–6), suspended in 10 μl sterile PBS. Vehicle control experiments used dilutions of drug solvent (DMSO) in PBS identical to those for drug dilutions.

Generation of rPce. pPce protein was produced as described previously (56), with modifications. pce was cloned from the P1121 S. pneumoniae genome with primers that introduced flanking restriction sites for BamHI and SacI (New England BioLabs Inc.) (see Supplemental Table 1). After restriction digestion and gel purification, the pce fragment was cloned into pET28a (Novagen) using T4 DNA ligase (New England BioLabs Inc.) to generate a construct in which pce was flanked with N- and C-terminal hexa-histidine tags. Ligated product was transformed into XL-1 Blue competent E. coli (Agilent), amplified, and purified by plasmid miniprep (Sigma-Aldrich). After sequence confirmation, pce-pET28a was transformed into BL21 (DE3)pLyS5 competent cells (Promega), and protein expression was induced according to the manufacturer’s instructions with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). Native cell lysis was achieved by sonication on ice, and lysates were applied to a Ni-NTA purification column (GE Healthcare Life Sciences) by fast protein LC (FPLC), followed by dialysis against 20 mM HEPES with 5 mM zinc sulfate.

Chromogenic assay for ChoP hydrolysis. Pce catalytic activity was assessed by incubation of 4 μg enzyme with 21 μg of the chromogenic substrate pNPPC (Sigma-Aldrich) at 37°C in 200 μl. Absorbance at 415 nm was monitored at the indicated time points (Figure 5C and Results) to detect ChoP hydrolysis and the resultant liberation of p-nitrophenol (56). For chromogenic assays using whole bacteria and isogenic mutants, 50 μl OD620 1.0 normalized bacteria was resuspended in potassium phosphate buffer (pH 8.0) and incubated with pNPPC, as above, for 120 minutes. For reactions testing H. influenzae strains, 1 U shrimp alkaline phosphatase (Affymetrix) was included in the assays to cleave terminal phosphate groups remaining after GlpQ hydrolysis.

Isolation of neutrophils from murine BM and human peripheral blood. Mature murine neutrophils were flushed from murine femora and washed with Hank’s Balanced Salt Solution buffer with calcium (Mediatech) containing 0.1% gelatin before enrichment with a discontinuous gradient of Histopaque-1077 and -1119 (Sigma-Aldrich). Flow cytometry confirmed that more than 90% of the CD45+ cells isolated were Ly6G+CD11b-. Human neutrophils were obtained from healthy donors and isolated by Polymorphrep (Axis Shield) gradient centrifugation as described previously (91).

Neutrophil activation and bactericidal assays. To assess the impact of Pce on PAF-stimulated neutrophil function, we performed assays using murine and human neutrophils ex vivo. Purified PAF (Cayman Chemical) was incubated in the presence or absence of 4 μg recombinant Pce in 100 μl potassium phosphate buffer (100 mM, pH 7.4) for 20 minutes at 37°C. Reaction products were applied to 104 murine or 105 human freshly isolated neutrophils at a 1:1 dilution and allowed to incubate for 30 minutes. All values listed for PAF represent final concentrations after incubation with neutrophils. Murine neutrophil activation was assessed by flow cytometric analysis of CD11b and CD64 as described above. Assays for neutrophil opsonophagocytic killing were performed as described previously (55, 84); 105 bacteria were preopsonized for 30 minutes with baby rabbit serum (BRS) as a complement source and applied to PAF-treated neutrophils at the indicated bacterium/phagocyte ratios (Figure 5, A, B, and D, and Figure 7H). Refer to the Supplemental Methods for details on neutrophil bactericidal assays involving H. influenzae.

Statistics. All data are presented as the mean ± SEM. Data were analyzed using a 2-tailed Student’s t test for comparisons between 2 groups and ANOVA with Newman-Keuls post test for all comparisons of more than 2 groups. Relative mean fluorescence intensity (MFI) measurements were analyzed using pairwise 1-sample Student’s t tests relative to a null ratio of 1. For all analyses, P < 0.05 was considered statistically significant.

Study approval. All animal experiments were approved by and performed in strict accordance with the guidelines of the IACUC of the University of Pennsylvania.

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