Supplemental Figures

Figure S1

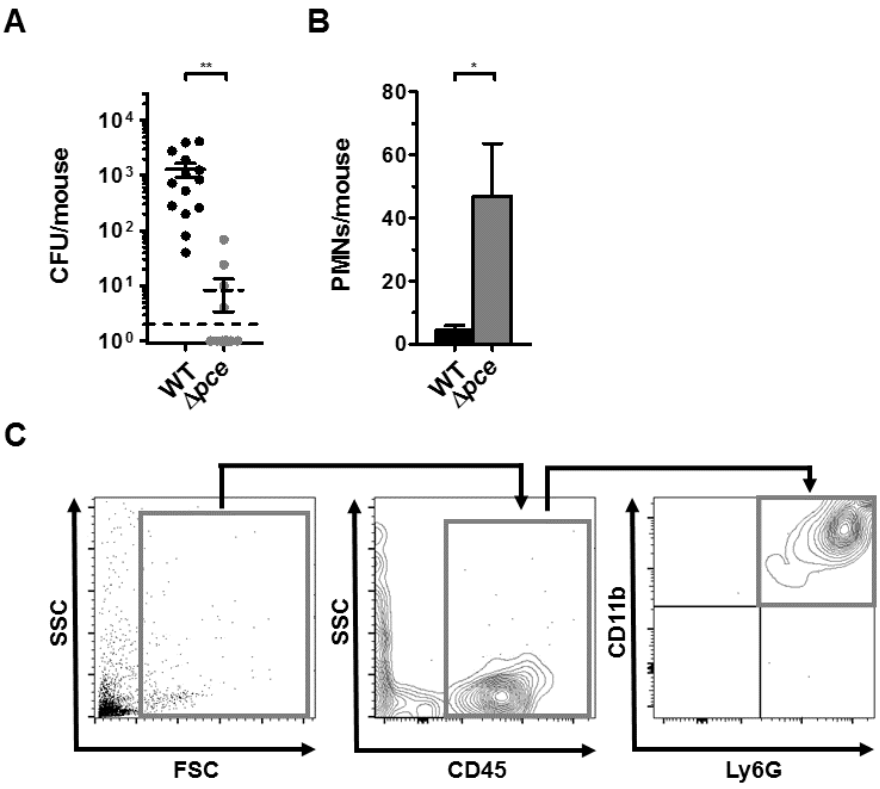

Figure S1. Clearance of Pce-deficient pneumococci on day 14 post-inoculation (p.i.) and upper airway luminal neutrophil gating strategy.
(A) Enumeration of WT (black) or Δ*pce* (grey) P1121 pneumococcal CFUs obtained from nasal lavages on day 14 p.i. (N = 14). (B) Quantification of luminal neutrophils elicited WT or Δ*pce* pneumococci on day 14 p.i. (N = 5). (C) Gating strategy used to identify $CD45^{+}CD11b^{+}Ly6G^{+}$ neutrophils from infiltrating immune cells during upper airway infection. Representative data represents neutrophils elicited by Δ*pce* pneumococci on day 4 p.i. Statistical significance was assessed by Student's *t* test. NS = not significant, * = $p < 0.05$.

Figure S2

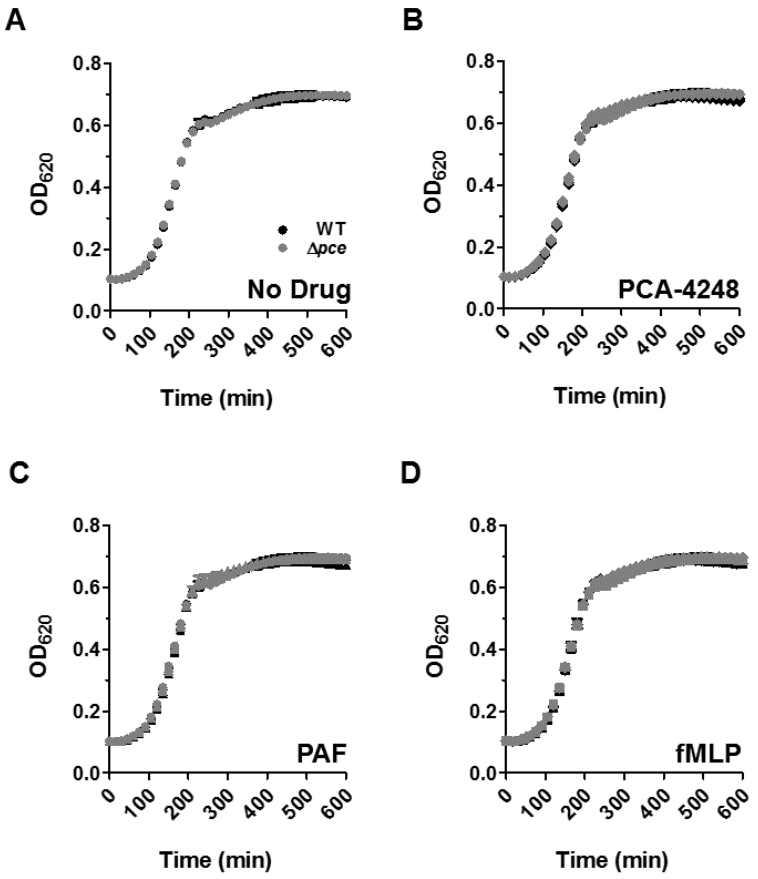

Figure S2. Assessment of the direct effects of PCA-4248, PAF, and fMLP on pneumococcal growth and viability *in vitro*.
Growth curves (OD_{620}) of WT (black) and Δ*pce* (grey) P1121 pneumococci in tryptic soy (TS) broth and grown either alone (A) or with increasing concentrations of PCA-4248 (B), PAF (C), or fMLP (D). Concentrations of drugs: PBS vehicle (circles), 2 nM (squares), 0.2 µM (diamonds), and 200 µM (triangles). Values represent three independent experiments. Statistical significance was assessed by Student's *t* test; all comparisons were not significant.

Figure S3

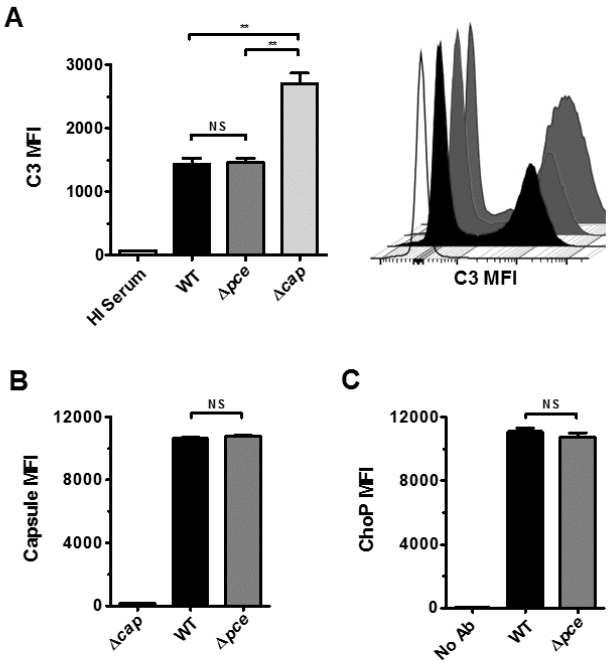

Figure S3. Quantification of complement deposition, capsule expression, and phosphorylcholine (ChoP) levels by bacterial flow cytometry.
Graphs and representative histograms for mean fluorescence intensity (MFI) of: (A) Complement component C3 deposited on WT (black) and Δ*pce* (grey) P1121 pneumococci after 30 minutes incubation with 66% murine serum. Experiments were also performed using heat-inactivated (HI) murine serum (white) and P1121Δ*cap* lacking capsule expression (light grey). (B) P1121 capsule expression on WT and Δ*pce* P1121 measured by deposition of anti-23F capsular antibody. (C) Accessibility of surface ChoP among P1121Δ*cap* and P1121Δ*pce*Δ*cap* pneumococci measured by deposition of anti-phosphorylcholine antibody. Values represent duplicated independent experiments. Statistical significance was assessed by one-way ANOVA with Newman-Keuls post-test. NS = not significant, ** = $p < 0.01$.

Table S1: Primers used in this study

	Description	Sequence (5' to 3')	Reference
***pce* amplification primers**	P1, F	GGGGAATTCAGAATGAAAAAGAAATTAAC	(1)
	PCE-2, R	TATGGATCCCTACTGTTCTGATTCCGATTTGTTTAC	(1)
Janus cassette insertion into *pce*			
	SP_0930 Upstream, F	TCTGCGTCCAGGGATTGTTC	This Study
	SP_0930 Upstream, R	CATTATCCATTAAAAATCAAACGCATCACTGCCACCTTCTTG	This Study
	Janus Cassette, F	CAAGAAGGTGGCAGTGATGCGTTTGATTTTTAATGGATAATG	(2)
	Janus Cassette, R	CACTCGTTTCAGAGCTTTGTGACTTTCCTTATGCTTTTGGAC	(2)
	SP_0930 Downstream, F	GTCCAAAAGCATAAGGAAAGTCACAAAGCTCTGAAACGAGTG	This Study
	SP_0930 Downstream, R	AAGGTCTGCTTGGACCATCG	This Study
Unmarked *pce* deletion			
	SP_0930 Upstream Del., F	GTCCAGGGATTGTTCACCGT	This Study
	SP_0930 Upstream Janus Fusion, R	GAAGGCCCTAAAACCTTCTTTTCTATCTTACTTTCTCAATCTCTCCAAGGTTTCC	This Study
	SP_0930 Downstream Janus Fusion, F	GGAAACCTTGGAGAGATTGAGAAAGTAAGATAGAAAAGAAGGTTTTAGGGCCTTC	This Study
	SP_0930 Downstream Del., R	CTTCCAACAAAAGCCCCTGC	This Study
***pce* cloning**			
	Pce cloning primer, F (BamHI site)	ATCGGATCCGCACTTGTAGGCGCTTTT	This Study
	Pce cloning primer, R (SacI site)	ATCGAGCTCACACTCGTTTCAGAGCTTTGTG	This Study
qRT-PCR	Lyso-PAF Acetyltransferase, F	GCCCAGGTGGCTTTCATGACGT	(3)
	Lyso-PAF Acetyltransferase, R	CCAGCAAACCACATGGTGCGC	(3)
	PAF Receptor, F	CTGGACCCTAGCAGAGTTGG	(4)
	PAF Receptor, R	GCTACTGCGCATGCTGTAAA	(4)
	CXCL1, F	CTGGGATTCACCTCAAGAACATC	(5)
	CXCL1, R	CAGGGTCAAGGCAAGCCTC	(5)
	CXCL2, F	CGCTGTCAATGCCTGAAG	(6)
	CXCL2, R	GGCGTCACACTCAAGCTCT	(6)
	GAPDH, F	TGCACCACCAACTGCTTAG	(5)
	GAPDH, R	GGATGCAGGGATGATGTTC	(5)

Supplemental Methods

Generation of an insertion-duplication Δpce mutant. A PCR product containing *pce* interrupted by an erythromycin resistance cassette was amplified from the R36A*Δpce S. pneumoniae* genome (a gift from Dr. Alexander Tomasz) using primers "P1" and "PCE-2" (see Table S1) (1). The product was transformed into the indicated wild type isolates, selected with 1 µg/mL erythromycin. Correct cassette insertion was verified by sequencing and lysates from all transformed mutants were incubated for 60 minutes with chromogenic p-nitrophenylphosphorylcholine to ensure no Pce enzymatic activity remained (see Methods).

Generation and correction of an in-frame Δpce deletion mutant. Deletion of *pce* was achieved through insertion and subsequent deletion of a bicistronic Janus construct bearing kanamycin resistance and streptomycin trans-sensitivity cassettes in spontaneously streptomycin-resistant bacteria, as detailed previously (2, 7). To insert the Janus cassette, an overlap-extension PCR product was created from three independent PCR reactions: First, a ~650 bp fragment upstream of *pce* was amplified from the pneumococcal genome using primers "SP_0930 Upstream, F&R"; the ~1.8 kb Janus cassette was amplified using primers "Janus Cassette, F&R"; and a ~640 bp fragment downstream of *pce* was amplified from the genome using primers "SP_0930 Downstream, F&R" (See Table S1). All three PCR products were then column-purified together (Qiagen) and extended using shared homology in a PCR reaction lacking primers. A final PCR using outside flanking primers "SP_0930 Upstream, F" and "SP_0930 Downstream, R" was performed to generate a product of approximately 3 kb. After purification, the product was transformed into wild type pneumococci and transformants were selected with 500 µg/mL kanamycin before sequence verification. A similar procedure was performed to create a construct to delete the Janus-bearing *pce* gene, wherein 2 PCR products were generated by: 1) "SP_0930 Upstream Del., F" & "SP_0930 Upstream Janus Fusion, R"; and 2) "SP_0930 Downstream Janus Fusion, F" & "SP_0930 Upstream Del., R." These

products were sewn together as described above and used to transform Janus-bearing *pce* mutants. Clones were selected with 500 µg/mL streptomycin and sequenced to confirm in-frame deletion of *pce.* Lastly, a genetically corrected (***Δpce::pce***) strain was generated by transforming Janus-bearing ***Δpce*** mutants with a ~3 kb PCR product containing wild type *pce* using primers "SP_0930 Upstream, F" and "SP_0930 Downstream, R" before selection with 500 µg/mL streptomycin and sequence verification.

In vitro bacterial growth and toxicity assays. Kinetic bacterial growth assays were performed as described previously (8); 96-well plates bearing 190 µL of TS broth supplemented with 10 µL catalase per well (30,000 U/mL) (Worthington Biochemical) were inoculated with wild type or ***Δpce*** pneumococci at OD_{620} 0.1. Bacterial growth and viability were monitored at 15-minute intervals over 10 hours in the presence of PBS (vehicle control), PAF (Cayman Chemical), PAF receptor antagonist PCA-4248 (Tocris), or fMLP (Sigma-Aldrich) at the indicated concentrations.

Complement deposition assays. Complement component C3 deposition assays were adapted from Dalia *et al*., 2010 (9). Approximately ~10^6 PBS-washed, mid-log-phase bacteria were incubated at 37° C for 30 minutes in 66% fresh murine serum (in Hank's Buffer with calcium). After a subsequent wash, bacteria were stained with 1:150 FITC-conjugated, monoclonal anti-mouse complement component C3 antibody (Accurate Chem.) and detected by flow cytometry. Assays were repeated with serum heat-inactivated for 60 minutes at 65° C as negative controls and bacteria lacking capsule served as positive controls for C3 deposition.

Quantification of bacterial capsule and surface phosphorylcholine. To quantify pneumococcal polysaccharide capsule by flow cytometry, PBS-washed bacteria were incubated with 1:500 Type 23F polyclonal typing sera (Statens Serum Institut) for 30 minutes, washed, then stained with 1:150 AF647-conjugated anti-rabbit IgG (Life Technologies). Capsule-deficient pneumococci served as negative controls. To quantify surface phosphorylcholine, WT and ***Δpce*** bacteria lacking capsule (***Δcap)*** were incubated as above with 1:500 monoclonal anti-

phosphorylcholine IgA (TEPC-15, Sigma-Aldrich) before staining with 1:100 FITC-conjugated anti-mouse IgA (Sigma-Aldrich).

H. influenzae neutrophil bactericidal assays. Bacterial killing assays were performed to assess whether pre-incubation of PAF with GlpQ phosphodiesterase inhibits PAF-stimulated neutrophil bactericidal function. Increasing concentrations of PAF were pre-incubated for 60 minutes with 25 µL heat-killed WT or *ΔglpQ H. influenzae* re-suspended in PBS from OD_{620} 1.0-normalized culture. Conditioned PAF solution was then centrifuged at 3,000*g* for 10 minutes to isolate supernatants, which were plated to confirm the absence of viable bacteria. Conditioned media from each condition was then applied to bone marrow-derived mature murine neutrophils, 10^5 phagocytes per reaction. After an additional 30-minute incubation, 10^2 CFU wild type *H. influenzae* (pre-opsonized in 66% baby rabbit serum) was added to the treated neutrophils in each reaction well. Bacterial survival was quantified in each condition by dilution plating after 45 minutes of incubation and compared to controls lacking neutrophils or PAF in pre-incubation steps.

Supplemental References

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