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HOST RESPONSE AND INFLAMMATION

Cytosolic Phospholipase $A_2\alpha$ Promotes Pulmonary Inflammation and Systemic Disease during *Streptococcus pneumoniae* Infection

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ABSTRACT Pulmonary infection by *Streptococcus pneumoniae* is characterized by a robust alveolar infiltration of neutrophils (polymorphonuclear cells [PMNs]) that can promote systemic spread of the infection if not resolved. We previously showed that 12-lipoxygenase (12-LOX), which is required to generate the PMN chemoattractant hepoxilin A_3 (HXA_3) from arachidonic acid (AA), promotes acute pulmonary inflammation and systemic infection after lung challenge with *S. pneumoniae*. As phospholipase A_2 (PLA_2) promotes the release of AA, we investigated the role of PLA_2 in local and systemic disease during *S. pneumoniae* infection. The group IVA cytosolic isoform of PLA_2 ($cPLA_2\alpha$) was activated upon *S. pneumoniae* infection of cultured lung epithelial cells and was critical for AA release from membrane phospholipids. Pharmacological inhibition of this enzyme blocked *S. pneumoniae*-induced PMN transepithelial migration *in vitro*. Genetic ablation of the $cPLA_2$ isoform $cPLA_2\alpha$ dramatically reduced lung inflammation in mice upon high-dose pulmonary challenge with *S. pneumoniae*. The $cPLA_2\alpha$-deficient mice also suffered no bacteremia and survived a pulmonary challenge that was lethal to wild-type mice. Our data suggest that $cPLA_2\alpha$ plays a crucial role in eliciting pulmonary inflammation during pneumococcal infection and is required for lethal systemic infection following *S. pneumoniae* lung challenge.

KEYWORDS *Streptococcus pneumoniae*, inflammation, neutrophils, phospholipase

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Streptococcus pneumoniae (the pneumococcus) is a Gram-positive pathogen that is capable of asymptomatically colonizing the nasopharynx of both children and adults. This organism can cause acute infections (sinusitis and otitis media) and is the most common cause of life-threatening community-acquired bacterial pneumonia (1, 2). Pneumococci are protected from clearance in the blood by an antiphagocytic polysaccharide capsule and other protective virulence factors; therefore, their entry into the circulation can lead to potentially lethal septicemia (3). Approximately 14.5 million cases of invasive pneumococcal disease occur annually worldwide, resulting in 0.5 to 1 million deaths of children less than 5 years old (4; http://worldpneumoniaday.org/wp-content/uploads/2014/10/Pneumococcal_factsheet.pdf).

A hallmark of pneumococcal pneumonia is a robust recruitment of neutrophils (polymorphonuclear cells [PMNs]) into the alveolar spaces (5, 6). Although PMN recruitment to the site of pathogenic insult is an integral part of innate immune defense, prolonged and robust PMN recruitment can contribute to disease and mortality (7–10).

Although the potent antimicrobial activities of PMNs, which include production of reactive oxygen species, proteases, cationic peptides, and inflammatory mediators, help contain infection, the poorly regulated release of these factors after PMN accumulation in the lungs leads to tissue destruction and potentially to lung failure (8, 11, 12).

PMN recruitment to the alveolar mucosal surface is a complex multistep process involving interactions between PMNs and endothelial, interstitial, and epithelial cells, cytokines, and various PMN chemokines and chemoattractants (7, 13–23). Eicosanoids are bioactive lipids that play critical roles in this inflammatory process (14, 17, 22, 23). Arachidonic acid (AA), the precursor of eicosanoids, is acted upon by cyclooxygenases (COX) to produce prostaglandins and thromboxanes or by lipoxygenases (LOX) to produce leukotrienes, lipoxins, and hepoxilins (24–26). We previously showed that the 12-LOX pathway and its products are important for PMN transepithelial migration during *S. pneumoniae* infection (27). This observation is consistent with other studies that have shown the 12-LOX pathway to be critical for PMN transepithelial migration across pulmonary epithelia during *Pseudomonas aeruginosa* infection and across *in vitro* intestinal epithelia during infection by *Salmonella enterica* serovar Typhimurium, *Shigella flexneri*, or enteroaggregative *Escherichia coli* (28–33).

The bulk of AA in mammalian cells is generated from the fatty acyl chains of glycerophospholipids present in cell membranes (34–36). AA availability is a rate-limiting factor in the production of eicosanoids, because AA release from membrane phospholipids due to enhanced activity of phospholipase A_2 (PLA_2) results in the increased production of eicosanoids (14, 37). AA is generated in various cell types by the action of PLA_2, which releases AA attached to the *sn-2* position of membrane phospholipids, or by diacylglycerol (DAG) lipase, which generates AA from diacylglycerols (37–43). Several inflammatory stimuli, such as the extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (p38 MAPK), c-Jun-NH2-terminal kinase (JNK) and tumor necrosis factor alpha (TNF-α), enhance PLA_2 activity, suggesting that PLA_2 may play an important role at the initiation of inflammation (44, 45).

The PLA_2 family includes at least three major subtypes: secretory PLA_2 ($sPLA_2$), calcium-independent PLA_2 ($iPLA_2$), and cytosolic PLA_2 ($cPLA_2$) (39, 42). The activity of $sPLA_2$ depends on millimolar concentrations of calcium, while that of $iPLA_2$, which is also cytosolic in nature, is calcium independent (39, 46). Of these subfamilies, the $cPLA_2$s are thought to be required for AA generation, as they have a substrate preference for phospholipids with AA at the *sn-2* position (14, 39, 40, 47). This enzyme is abundantly expressed in multiple tissue and cell types, including human lungs and alveolar epithelial cells, and has the highest transcript levels in the lungs, brain, kidneys, heart, and spleen (48, 49). Numerous inflammatory stimuli, such as interleukin 1β (IL-1β), gamma interferon (IFN-γ), and TNF-α, trigger the release of eicosanoids from human lung epithelial cells in a manner dependent on $cPLA_2$ activation (45, 50, 51). $cPLA_2$ has been implicated in PMN recruitment *in vitro* and promotes sepsis-induced acute lung injury (36, 52, 53), but its role in *S. pneumoniae*-induced lung inflammation and subsequent systemic spread of the pathogen has yet to be defined. Here we show that $cPLA_2\alpha$ plays a critical role in *S. pneumoniae*-induced AA release from cultured human airway epithelial cells and subsequent PMN transepithelial migration and is required for pulmonary inflammation, bacteremia, and lethality upon pneumococcal lung infection of mice.

RESULTS

AA is released from membrane phospholipids during *S. pneumoniae* infection of cultured airway epithelial cells. Robust recruitment of PMNs into alveolar spaces is a hallmark of pneumococcal pneumonia, and we previously showed that blocking the 12-LOX pathway abrogated PMN migration response across pulmonary epithelia (27). As arachidonic acid (AA) is the substrate for 12-LOX activity, we sought to determine whether pneumococcal infection leads to AA release from pulmonary epithelial cells. We incorporated [^{3}H]AA into the membrane phospholipids of NCI-H292 (H292) cell monolayers, and following infection with the *S. pneumoniae* clinical isolate

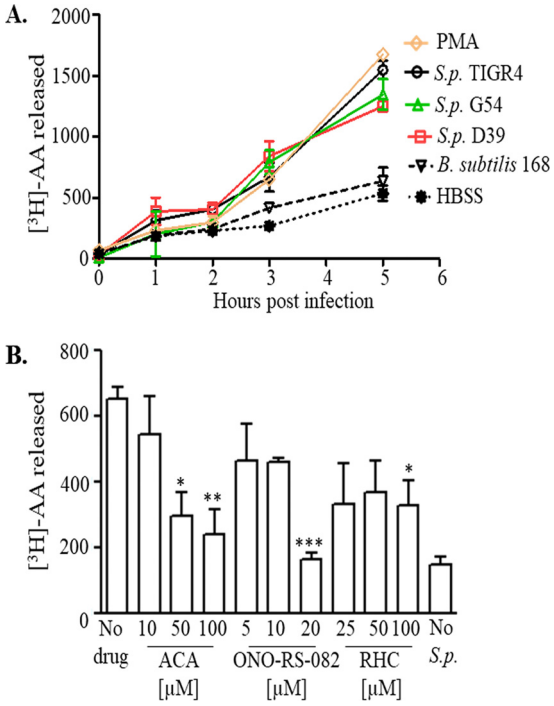

FIG 1 *S. pneumoniae* infection of airway epithelial cells triggers arachidonic acid (AA) release. (A) H292 cell monolayers were labeled with [^{3}H]AA as detailed in Materials and Methods and infected with the indicated *S. pneumoniae* (*S.p.*) strains (each of a different capsular serotype) or with *B. subtilis* strain 168 at a multiplicity of infection (MOI) of 10 (1 $\times 10^7$ CFU/monolayer). Supernatants were collected at the indicated times postinfection, and radioactive counts released into the supernatants were measured by scintillation counting. Labeled H292 cell monolayers treated with PMA and HBSS+Ca/Mg were used as positive and negative controls, respectively. Shown are results from a representative of two experiments. (B) H292 cell monolayers labeled with [^{3}H]AA were treated with the indicated concentrations of pan-PLA$_2$ inhibitors ACA or ONO-RS-082 or the DAG lipase inhibitor RHC-80267 (labeled as RHC) prior to infection with *S. pneumoniae* TIGR4, as detailed in Materials and Methods. Labeled H292 cell monolayers treated with HBSS+Ca/Mg were used as negative controls. Radioactive counts released into supernatants were determined by scintillation counting. Shown are results from a representative of three experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared with the no-drug control, using one-way ANOVA).

strains TIGR4, D39, and G54, we assessed AA release by scintillation counting of culture supernatants. Culture supernatants of monolayers treated with Hanks' balanced salt solution (HBSS) alone or the nonpathogenic Gram-positive bacterium *Bacillus subtilis* were used as negative controls, and the global signaling pathway activator phorbol myristate acetate (PMA), previously shown to induce AA release (54), was used as a positive control. Lactate dehydrogenase (LDH) release assays indicated that *S. pneumoniae* infection was not cytotoxic to H292 cells over the periods tested (see Table S1 in the supplemental material), but infection resulted in a significant increase in AA release from membrane phospholipids in a time-dependent manner, with no significant differences observed among the *S. pneumoniae* strains tested (Fig. 1A). Infection with *B. subtilis* resulted in significantly less release of AA than with the *S. pneumoniae* strains (2.5-fold lower than with strain TIGR4 and 2-fold lower than with strains D39 and G54; at 5 h postinfection, $P < 0.005$, $P < 0.05$, $P < 0.05$, respectively) and was statistically indistinguishable from treatment with HBSS. Strain TIGR4 (55) was previously used for monolayer and murine infection experiments (27, 56), including in investigations that revealed 12-LOX-dependent inflammation, and thus was utilized for the studies described below.

cPLA$_2$ has a high specificity for membrane phospholipids with AA at the *sn-2* position, and its activation has been implicated in AA release and inflammation (54, 57). It is also known that diacylglycerol (DAG) lipase, in combination with monoacylglycerol lipase or fatty acid amidohydrolase, can result in AA release from DAG (43). To assess

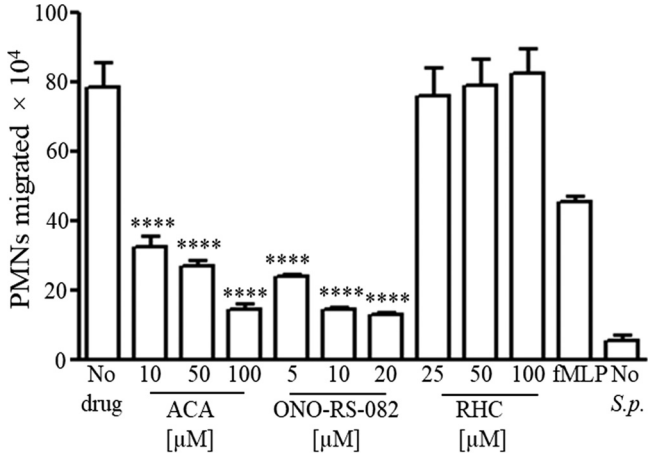

FIG 2 PLA_2 is critical for *S. pneumoniae*-elicited PMN transepithelial migration. H292 cell monolayers were treated with different concentrations of pan-PLA_2 inhibitors ACA and ONO-RS-082 or the DAG lipase inhibitor RHC-80267 prior to infection with *S. pneumoniae* TIGR4, as detailed in Materials and Methods. PMNs were added basolaterally, and the number of PMNs that migrated to the apical side was calculated using the myeloperoxidase (MPO) assay. Monolayers treated with fMLP and HBSS+Ca/Mg were used as positive and negative controls, respectively. Shown are results from a representative of three experiments. ****, $P = 0.0001$ compared to the no-drug control, using one-way ANOVA.

the potential role of PLA_2 and DAG lipases in AA release during *S. pneumoniae* infection, we treated the [^{3}H]AA-labeled H292 cell monolayers with various concentrations of the pan-PLA_2 inhibitors ONO-RS-082 and *N*-(*p*-amylcinnamoyl) anthranilic acid (ACA) (50% inhibitory concentration [IC_{50}s] = 9.3 and 10.6 μM, respectively) or the DAG lipase-specific inhibitor RHC-80267 (IC_{50} = 5 μM) prior to infecting the cells with *S. pneumoniae* TIGR4 (41, 58–60). RHC-80267 inhibited AA release 50% compared with that in untreated monolayers, but we detected no dose-response relationship and the maximal degree of inhibition was 50% (Fig. 1B). In contrast, the PLA_2 inhibitors significantly ($P < 0.05$) reduced the amount of [^{3}H]AA released from TIGR4-infected H292 cells in a dose-dependent manner, with a maximal reduction of 63 to 75% (Fig. 1B). Note that none of the inhibitors was cytotoxic to the H292 cells (see Table S3) or interfered with bacterial growth or binding to monolayers (see Fig. S1A and B, respectively).

cPLA$_2$ is critical for eliciting PMN transepithelial migration during pneumococcal infection. Since AA is the precursor of a number of proinflammatory eicosanoids, including the PMN chemoattractant HXA_3 (17, 30), we inferred that increased levels of AA release may play a role in PMN transepithelial migration. To determine whether AA release by PLA_2 or DAG lipases was required for TIGR4-induced PMN migration *in vitro*, we treated H292 cell monolayers with the pan-PLA_2 or DAG lipase inhibitors prior to *S. pneumoniae* infection and measured subsequent PMN transepithelial migration. Pretreatment with the DAG lipase inhibitor did not significantly reduce PMN migration (Fig. 2). In contrast, the pan-PLA_2 inhibitors resulted in a dose-dependent reduction in PMN transepithelial migration compared with that in untreated H292 cell monolayers, with a maximal inhibition of more than 80%. The specificity of this response was demonstrated by the observation that the pan-PLA_2 inhibitors each failed to inhibit fMLP-induced PMN transepithelial migration (see Fig. S1C). Additionally, these inhibitors had no effect on H292 cell barrier properties at the doses used (see Table S4). This result suggests that the AA released during *S. pneumoniae* infection is derived from membrane phospholipids, a substrate of PLA_2, rather than from diacylglycerols.

$cPLA_2$ has a high specificity for membrane phospholipids with AA at the *sn-2* position (39), and its activation has been implicated in the release of AA and inflammation (54, 57). This enzyme is activated upon serine phosphorylation by MAP kinases, leading to its translocation to the plasma membrane and catalysis of phospholipids (42, 50). To determine whether *S. pneumoniae* infection of lung epithelial cells leads to the phosphorylation and membrane localization of $cPLA_2$, we infected H292 cell

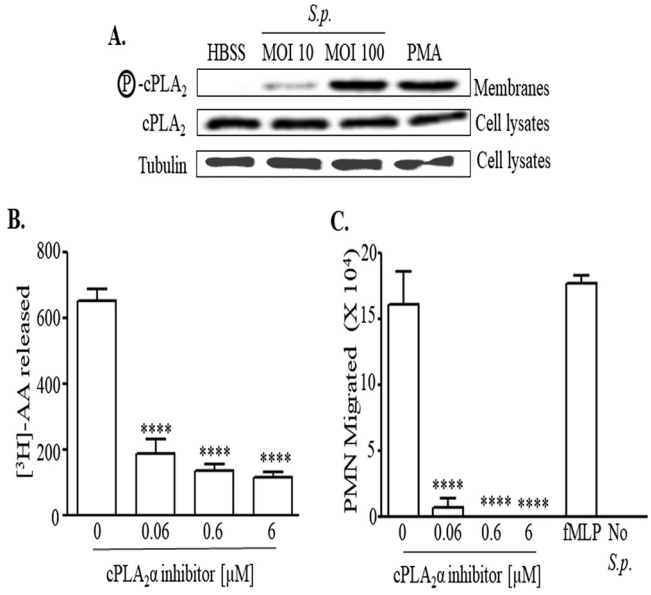

FIG 3 cPLA$_2$ is activated upon pneumococcal infection of pulmonary epithelial monolayers and is critical for eliciting AA release and PMN transepithelial migration. (A) H292 cell monolayers were infected with *S. pneumoniae* TIGR4 at an MOI of 10 or 100. Monolayers treated with PMA and HBSS+Ca/Mg were used as positive and negative controls, respectively. Cell membranes were prepared as described in Materials and Methods and were probed with anti-phospho-cPLA$_2$, anti-cPLA$_2$, or antitubulin antibody. Shown are results from a representative of two independent experiments. (B) H292 cell monolayers labeled with [^{3}H]AA were treated with the indicated concentrations of cPLA$_2\alpha$ inhibitor prior to infection with *S. pneumoniae* TIGR4, as detailed in Materials and Methods. Radioactive counts in supernatants were measured by scintillation counting. Shown are results from a representative of three experiments. ****, $P = 0.0001$ compared to the untreated control, using one-way ANOVA. (C) H292 cell monolayers were treated with the indicated concentrations of the cPLA$_2\alpha$ inhibitor prior to infection with *S. pneumoniae* TIGR4, as detailed in Materials and Methods. PMNs were added basolaterally, and the number of PMNs that migrated to the apical side was calculated using the MPO assay. Monolayers treated with fMLP and HBSS+Ca/Mg were used as positive and negative controls, respectively. Shown are results from a representative of three experiments. ****, $P < 0.0001$ compared to the respective no-drug controls, using one-way ANOVA.

monolayers with *S. pneumoniae* and performed immunoblotting to determine both total amount of cPLA$_2$ in cell lysates and the amount of phosphorylated cPLA$_2$ in cell membrane preparations. PMA, a known activator of cPLA$_2$ (61), was included as a positive control. We found that *S. pneumoniae* infection resulted in no change in total cPLA$_2$ but also a dose-dependent activation of cPLA$_2$, evidenced by increasing amounts of phosphorylated cPLA$_2$ in membrane fractions (Fig. 3A).

Of the six isoforms of cPLA$_2$ (39), cPLA$_2\alpha$ is crucial for eicosanoid production in the airway epithelium (42, 45, 50, 62), and we took advantage of a potent ($IC_{50} = 0.05\ \mu M$) and highly specific cPLA$_2\alpha$ inhibitor (63) to further investigate the role of cPLA$_2\alpha$ in generating AA during pneumococcal infection. This inhibitor was not cytotoxic to H292 cells (see Table S3) and did not affect bacterial growth and binding to monolayers (Fig. S1A and B, respectively). Nevertheless, treatment of [^{3}H]AA-labeled H292 cell monolayers with as little as a 0.06 μM concentration of this inhibitor prior to *S. pneumoniae* infection reduced AA release 3.5-fold ($P = 0.001$ [Fig. 3B]) and almost completely inhibited PMN transepithelial migration compared with that in untreated H292 monolayers ($P < 0.005$ [Fig. 3C]), strongly suggesting that cPLA$_2\alpha$ plays a critical role in inflammatory signaling during *S. pneumoniae* infection.

cPLA$_2\alpha$ deficiency diminishes pulmonary inflammation in mice challenged with *S. pneumoniae*. We cannot rule out off-target effects of the cPLA$_2$ inhibitors utilized as described above or that the immortalized H292 cells fully reflect the response of bona fide pulmonary epithelium. Hence, to test the role of cPLA$_2\alpha$ in pneumococcal disease, we utilized wild-type (WT) or *cPLA$_2\alpha^{-/-}$* BALB/c mice (64). Basal levels of pulmonary dendritic cells, macrophages, and T cells, measured by flow cytometry of single-cell

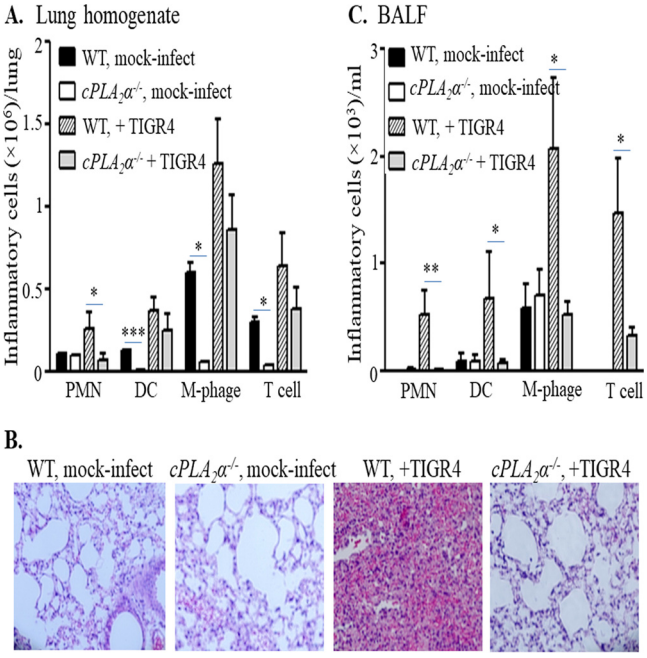

FIG 4 cPLA$_2\alpha$ promotes pulmonary inflammation in *S. pneumoniae*-infected mice. Mice (*cPLA*$_2\alpha^{-/-}$ or their WT littermates) were mock infected with PBS ($n = 4$ for each strain, per experiment) or infected intratracheally with *S. pneumoniae* TIGR4 ($n = 5$ for each strain, per experiment), as detailed in Materials and Methods. Lungs and bronchoalveolar lavage fluid (BALF) were collected from the mock-infected or TIGR4-infected mice. Cells present in the digested lungs and BALF were stained with relevant MAbs, and the fluorescence intensities of the stained cells were determined by flow cytometry. Collected data were analyzed to determine the numbers of PMNs, dendritic cells (DC), macrophages (M-phage), or T cells in the lungs (A) and in the BALF (C). Statistical significance was analyzed by one-way ANOVA followed by individual Student's *t* test analyses. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$. For histological analyses (B), H&E-stained lung sections were prepared and examined by light microscopy (original magnification, ×20). Shown are results from a representative of two independent experiments.

lung suspensions, were significantly lower in cPLA$_2\alpha$-deficient mice than in WT mice (Fig. 4A, "DC," "M-phage," and "T cell"), consistent with the lower baseline levels of eicosanoids, such as the proinflammatory leukotriene B_4 (LTB_4), associated with cPLA$_2\alpha$ deficiency (52).

Notably, striking differences between WT and *cPLA*$_2\alpha^{-/-}$ mice were observed upon intratracheal challenge with 2×10^5 CFU of *S. pneumoniae* TIGR4. Similar to our previous observation in C57BL/6 mice (27), wild-type BALB/c mice exhibited a robust acute inflammatory response at 48 h postinfection, with vascular congestion and massive cellular infiltrates (Fig. 4B, "WT, mock-infect" versus "WT, +TIGR4"). Flow cytometry of lung homogenates and/or bronchoalveolar lavage fluid (BALF) revealed greater numbers of PMNs, dendritic cells, macrophages, and T cells in infected mice than in uninfected controls (Fig. 4A and C). In contrast, infection of *cPLA*$_2\alpha^{-/-}$ littermates resulted in dramatically lower levels of pulmonary inflammation upon *S. pneumoniae* challenge (Fig. 4B, "WT, +TIGR4" versus "*cPLA*$_2^{-/-}$, +TIGR4"). Flow cytometric analyses of pulmonary homogenates showed that PMN recruitment to the lung was 2.5-fold lower than in WT mice, i.e., diminished to background (uninfected) levels (Fig. 4A). PMN recruitment to the airways was also dramatically affected, as virtually no PMNs were detected in BALF (Fig. 4C). Quantitation of PMNs in lung homogenates or BALF by myeloperoxidase (MPO), a lysosomal peroxidase present in the azurophilic granules of PMNs, also indicated an essential role for cPLA$_2\alpha$ in promoting acute inflammation following pulmonary challenge with *S. pneumoniae* TIGR4 (see Fig. S2A). Finally, the recruitment of dendritic cells, macrophages, and T cells to airway spaces, as assessed by flow cytometric analysis of BALF, was also significantly diminished by ablation of *cPLA*$_2\alpha$ (Fig. 4C), consistent with the lower levels of a variety of eicosanoids in cPLA$_2\alpha$-deficient

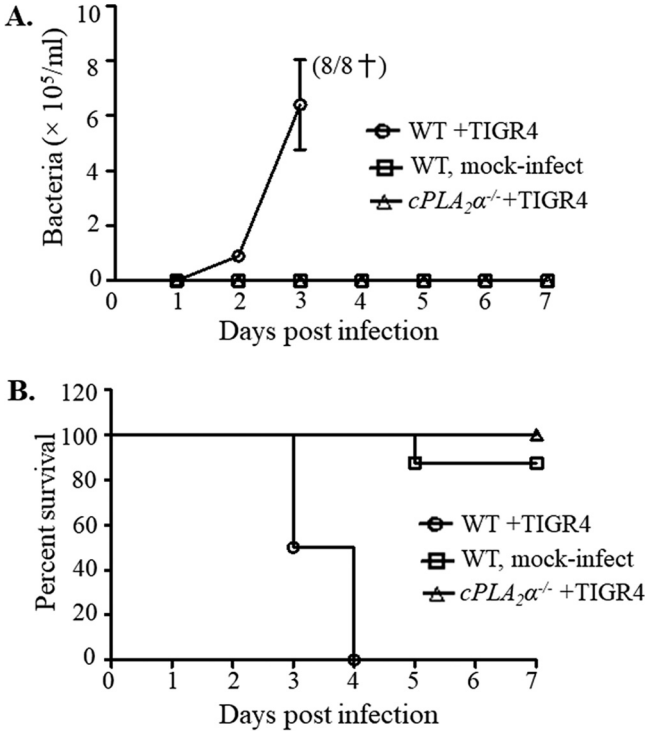

FIG 5 Ablation of *cPLA$_2\alpha$* leads to decreased bacteremia and increased survival in an otherwise lethal *S. pneumoniae* lung challenge. *cPLA$_2\alpha^{-/-}$* mice or their littermate WT controls were intratracheally inoculated with TIGR4 (see Materials and Methods). A control group of WT mice received PBS. (A) Bacteremia was determined by plating tail vein blood at the specified time points. Shown are results from a combination of two experiments. Death of all infected WT mice is indicated by a dagger. (B) Survival of animals was monitored over a 7-day postinfection period. The log rank (Mantel-Cox) test was performed for survival curve analysis. The experiment was performed twice, with similar results. Shown are results from a combination of two experiments. A total of 8 mice were used per group; panels A and B represent the same cohorts of mice.

mice in other lung injury models (52). Interestingly, the numbers of bacteria in either lung homogenates or BALF at 48 h postinfection were not significantly different between WT and *cPLA$_2\alpha^{-/-}$* mice (see Fig. S2B), consistent with previous findings that genetic ablation or chemical inhibition of 12-LOX, which is required for the production of HXA_3, did not result in an increase in bacterial load in the lung (27). These data indicate that cPLA$_2\alpha$ plays a critical role in the recruitment of PMNs and other inflammatory cells following *S. pneumoniae* lung infection.

Ablation of *cPLA$_2\alpha$* leads to decreased bacteremia and increased survival in an otherwise lethal *S. pneumoniae* lung challenge. Acute pulmonary inflammation during *S. pneumoniae* lung infection may disrupt epithelial barrier function and enhance tissue damage (10, 27, 65, 66), and we previously found that genetic ablation or chemical inhibition of 12-LOX mitigated bacteremia and lethality following intratracheal challenge with *S. pneumoniae* (27). To determine whether cPLA$_2\alpha$ is important for invasive pneumococcal disease, we infected *cPLA$_2\alpha^{-/-}$* mice and littermate BALB/c controls intratracheally with *S. pneumoniae* and monitored bacteremia, illness, and survival. At 48 h postinfection, ~10^5 CFU/ml were present in the tail vein blood of WT mice, and at 72 h postinfection, at which time bacteremia reached ~10^6 CFU/ml, all the WT mice succumbed to infection or were moribund and euthanized (Fig. 5). In contrast, infected *cPLA$_2\alpha^{-/-}$* mice suffered no detectable bacteremia and uniformly survived (Fig. 5).

DISCUSSION

Robust pulmonary infiltration of PMNs is a characteristic of pneumococcal lung infection (5, 6). Eicosanoids, which are formed from AA by the action of lipoxygenases,

cyclooxygenases, and/or cytochrome P450, are widely implicated in lung inflammation by *S. pneumoniae* and other bacteria (27, 31, 67). We previously showed that pneumococcus-mediated induction of 12-LOX, an enzyme essential for the conversion of AA to the neutrophil chemoattractant HXA_3, promotes basolateral-to-apical transepithelial recruitment of PMNs *in vitro*, as well as the corresponding apical-to-basolateral migration of bacteria (27). 12-LOX expression is increased during pneumococcal infection, and chemical inhibition or genetic ablation of 12-LOX protected mice from pulmonary inflammation and lethal systemic infection following intratracheal challenge with *S. pneumoniae* (27).

Pneumococcal infection triggers the release of AA and its metabolites in the middle ear during otitis media (68).We showed in this study that pneumococcal infection of respiratory epithelial monolayers also results in the release of AA. Both phospholipases and DAG lipases have been implicated in the generation of AA (41), and we found that a DAG inhibitor, at the highest concentration tested, decreased AA production 2-fold. However, PLA_2 has been shown to play a more important role in releasing AA in a variety of other physiologic and pathophysiologic processes (64), and two pan-PLA_2 inhibitors resulted in greater (3- to 4-fold) decreases in AA release. In addition, both PLA_2 inhibitors diminished PMN transepithelial migration by $>$80% during pneumococcal infection of respiratory epithelial monolayers, whereas the DAG lipase inhibitor had no effect. Although we cannot rule out a role for DAG in the production of eicosanoid PMN chemoattractants, PLA_2 has been previously shown to be activated by pneumococcal virulence factors, such as the pore-forming toxin pneumolysin, in pulmonary artery endothelial cells and human neutrophils (69, 70).

The PLA_2 superfamily, which cleaves the ester bond of phospholipids at the *sn-2* position to generate free fatty acids, includes at least three major subfamilies: $sPLA_2$s, $iPLA_2$s and $cPLA_2$s (39, 42). Of these, $cPLA_2$ is highly specific for phospholipids with AA at the *sn-2* position and thus plays an important role in the generation of free AA (39, 50, 62). $cPLA_2$ has been implicated in airway inflammation both in humans and mice (37, 71, 72). For example, lung epithelial cells of cystic fibrosis (CF) patients have elevated $cPLA_2$ activity and produce more eicosanoids than do normal epithelial cells (73). Activation of $cPLA_2$ via phosphorylation is imperative for the production of eicosanoids and is dependent on MAP kinases and protein kinase C (PKC) (42, 50, 74), and we found that phosphorylated $cPLA_2$ accumulated in the membrane fraction of lung epithelial cells after pneumococcal infection.

The $cPLA_2$ family consists of six members ($cPLA_2\alpha$, -β, -γ, -δ, -ε, and -ζ) (62). $cPLA_2\gamma$ is induced in eosinophils and pulmonary epithelial cells upon sensitization of mice with *Aspergillus fumigatus* extracts (75), but overall the role of the non-$cPLA_2\alpha$ members of the $cPLA_2$ family remains poorly characterized. In contrast, $cPLA_2\alpha$ has been shown to be crucial for eicosanoid production in a variety of tissues (45, 50, 62), including the human intestine (76, 77), and has been implicated in pulmonary disorders such as acute respiratory distress syndrome (ARDS), CF, and allergy (71, 78). For example, during ARDS, $cPLA_2\alpha^{-/-}$ mice display lower baseline levels of eicosanoids in BALF than do littermate control WT mice, as well as lower levels of neutrophil infiltration (52). We found that an inhibitor of $cPLA_2\alpha$ dramatically diminished AA release and virtually eliminated PMN migration across epithelial monolayers *in vitro*. Given the inherent limitations of *in vitro* chemical inhibitor studies, we utilized a murine model to assess the role of $cPLA_2\alpha$ and found that $cPLA_2\alpha$ deficiency (64) entirely blocked pneumococcus-induced PMN recruitment to the lung. $cPLA_2\alpha$ was also required for maximal recruitment of macrophages, dendritic cells, and T cells in the lung and airways upon infection, suggesting a more general role for $cPLA_2\alpha$ in pulmonary inflammation. The $cPLA_2\alpha$-dependent extravasation of these other inflammatory cell types may in part be a consequence of generalized compromise of integrity of the airway epithelial barrier resulting from transepithelial PMN migration (27). In addition, other AA metabolites function as inflammatory mediators for a variety of cells (79–81) such that the release of AA by $cPLA_2\alpha$ may promote the migration of several inflammatory cell types into airway spaces.

Previous reports have shown that in several models, PMNs are required for pneu-

mococcal control early after pulmonary challenge (82, 83) but may not promote bacterial clearance at later phases of infection (10, 27). We also did not observe an effect of cPLA$_2\alpha$-mediated inflammatory signaling on bacterial numbers in the lungs or BALF of mice at 48 h postinfection with pneumococcal doses included in this study, consistent with our previous observations that 12-LOX-deficient mice also suffered no greater pneumococcal loads in the lung and airways than did WT C57BL/6 mice (27; unpublished data).

Finally, pulmonary inflammation during pneumococcal infection contributes to epithelial barrier compromise and promotes systemic disease (65, 84, 85). We found that not only did cPLA$_2\alpha$ fail to promote bacterial clearance in the lung, but also this enzyme actively promoted lethal systemic disease: cPLA$_2\alpha$-deficient mice, like 12-LOX-deficient mice (27), were protected from *S. pneumoniae* bacteremia after pulmonary challenge and uniformly survived an otherwise lethal challenge. Our study supports the possibility that inhibition of cPLA$_2$-mediated PMN migration may provide a strategy to combat invasive pneumococcal infection. Given that cPLA$_2$-mediated inflammation appears central to other pulmonary disorders, such as ARDS, CF, and allergy (71, 78), these inhibitors may have broad therapeutic value for noninfectious disorders as well.

MATERIALS AND METHODS

Bacterial strains. *S. pneumoniae* strains TIGR4 (serotype 4 [55]), D39 (serotype 2 [86]), and G54 (serotype 19F [87]) were grown in Todd-Hewitt broth (BD Biosciences, San Jose, CA) supplemented with yeast extract at 37°C and 5% CO_2 and used in mid-log phase to late log phase. *Bacillus subtilis* strain 168 was grown overnight at 37°C in Luria broth. *S. pneumoniae* strain TIGR4 is highly virulent in mice and has been used in our previous murine infection studies, including those on 12-LOX-dependent pulmonary inflammation (27, 56). For the murine infection experiments, bacteria (5×10^8/ml) were stored in medium supplemented with 25% (vol/vol) glycerol at −80°C. Prior to use, the frozen cultures were thawed and diluted in phosphate-buffered saline (PBS). Bacterial stock titers were confirmed by plating serial dilutions on blood agar at 37°C and 5% CO_2. When required, *S. pneumoniae* strains were heat killed at 65°C for 1 h (27).

Growth, maintenance, and infection of epithelial cells. Human mucoepidermoid pulmonary carcinoma cell line NCI-H292 (H292) has been used in a number of studies of pulmonary inflammation and/or eicosanoid production (88–92), as well as in studies of pulmonary infection by bacteria (93–95). These cells were cultured in RPMI 1640 medium (American Type Culture Collection, Manassas, VA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin-streptomycin. Polarized H292 cell monolayers were grown on the underside of collagen (rat tail, type IV)-coated 0.33-cm^2 membrane filters (Corning Life Sciences, Tewksbury, MA). Cells were infected with bacteria resuspended in Hanks' balanced salt solution (HBSS) supplemented with Ca^{2+} and Mg^{2+} (HBSS+Ca/Mg), at 1×10^7 bacteria/monolayer, a multiplicity of infection of 10. The cytotoxicity of H292 cell monolayers in response to *S. pneumoniae* TIGR4 infection as measured by lactate dehydrogenase (LDH) release (30) is shown in Table S1 in the supplemental material. As the transepithelial resistance of lung epithelial monolayers is typically very low (96), permeability to horseradish peroxidase (HRP) was used to assess monolayer barrier integrity (30) and is shown in Table S2.

Animals. cPLA$_2\alpha$ knockout (*cPLA$_2\alpha^{-/-}$*) mice were generated in a BALB/c background using gene targeting in mouse embryonic stem cells to disrupt the exon containing Ser228, thus generating a null allele (64). The *cPLA$_2\alpha^{-/-}$* mice were backcrossed for 10 generations and genotyped using tail DNA. All animal experiments were performed with *cPLA$_2\alpha^{-/-}$* mice and their wild-type BALB/c littermate controls in accordance with Tufts University Animal Care and Use Committee-approved protocols.

PMN isolation and transmigration assay. PMNs were isolated from blood drawn from healthy human volunteers according to previously described protocols (30, 97). The PMN isolation protocol was approved by the Tufts University Institutional Review Board, and informed written consent was obtained from all volunteers. Basolateral-to-apical PMN transmigration assays were performed as described previously (27). Formyl-methionyl-leucyl-phenylalanine (fMLP) at 10^{-11} M or buffer alone (HBSS+Ca/Mg) was used as the positive or negative control, respectively, for induction of PMN transmigration.

AA release assay. Arachidonic acid (AA) release in epithelial cell culture was assayed using previously described protocols (37, 98). Briefly, H292 cells grown on 24-well tissue culture inserts were incubated in medium containing 0.2 μCi/ml of [^{3}H]AA (PerkinElmer, Waltham, MA) for 18 to 24 h prior to infection, to allow for the incorporation of [^{3}H]AA into membrane phospholipids. Cells were then washed in PBS and infected with 1×10^7 *S. pneumoniae* organisms resuspended in 0.5 ml of HBSS+Ca/Mg. Supernatants were collected at different time points and measured for radioactivity using a scintillation counter (Becton Dickinson). At the end of the experiment, H292 cells were lysed in 1% SDS and 1% Triton X-100 to determine total radioactivity.

Drug treatment. H292 cell monolayers were incubated with different doses of the pan-PLA$_2$ inhibitor ONO-RS-082 or *N*-(*p*-amylcinnamoyl) anthranilic acid (ACA) or the DAG lipase inhibitor RHC-80267 (Enzo Life Sciences, Farmington, NY) (38, 58). cPLA$_2\alpha$ was specifically inhibited in H292 cell monolayers by incubation with the cPLA$_2\alpha$ inhibitor (*N*-{(2*S*,4*R*)-4-(biphenyl-2-ylmethyl-isobutyl-amino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl}-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl]acrylamide), HCl (EMD Millipore, Billerica, MA), diluted to 0.06, 0.6, and 6 μM from a stock concentration of 6 mM in dimethyl

sulfoxide (DMSO). Working dilutions were made in HBSS+Ca/Mg from the stock solutions. Monolayers were incubated with inhibitors for 2 h at 37°C and then washed to remove the residual drug prior to infection. Vehicle controls were used to rule out nonspecific effects. The inhibitors did not affect cell viability or H292 cell barrier integrity, as assessed by LDH and HRP migration assays, and had no effect on bacterial growth or adherence to H292 cell monolayers.

Cell viability and bacterial binding assays. Cell viability in response to bacterial or drug treatment was measured by LDH assays as previously described (30). Bacterial binding was measured after incubating the bacteria for 40 min with H292 cell monolayers, using a previously described protocol (27).

Detection of cPLA$_2$ activation. H292 cell monolayers were infected with *S. pneumoniae* TIGR4 for 2.5 h. Control monolayers received buffer (HBSS+Ca/Mg) alone as a negative control or 1 μM phorbol myristate acetate (PMA) as a positive control. Membrane fractions were prepared as previously described (99). Equal amounts of protein were run on a 4 to 20% gradient polyacrylamide gel (Bio-Rad, Hercules, CA) and then transferred onto nitrocellulose membranes. Western blots were probed with anti-cPLA$_2$ or anti-phospho-cPLA$_2$ antibodies (Cell Signaling Technologies, Danvers, MA) and detected with ECL reagent. To verify equal sample loading, blots were probed with antitubulin antibody.

Murine infection studies. *cPLA$_2\alpha^{-/-}$* mice (64) and their littermate wild-type (WT) BALB/c controls were challenged with 2×10^5 *S. pneumoniae* TIGR4 organisms in 50 μl of PBS via intratracheal inoculation, whereas control mice received only PBS. For histological study of pulmonary inflammation, mice were euthanized at 48 h postinfection, and whole lungs were excised. Lungs were fixed in 10% buffered formalin (Sigma-Aldrich, St. Louis, MO) and embedded in paraffin. Lung blocks were sectioned at 5 μM and adhered to silanized slides. Three mice per group were analyzed following hematoxylin and eosin (H&E) staining, and lungs were visualized using a Nikon TMS microscope. PMN migration into the lung and airways was measured by myeloperoxidase (MPO) assay using a previously described protocol (100). To determine the bacterial load in the lungs at 48 h, lungs were isolated and homogenized in PBS, and serial dilutions were plated on blood agar. Serial dilutions of bronchoalveolar lavage fluid (BALF) were plated on blood agar to enumerate bacteria in the airways. For bacteremia, tail blood was collected and dilutions were plated on blood agar plates every 24 h postinfection. Mice were monitored for bacteremia, sickness, and survival over 7 days following infection, and moribund animals were euthanized as per the protocol approved by the Tufts University Animal Care and Use Committee.

Flow cytometry. Anti-Ly-6G-phycoerythrin (PE) (clone 1A8), anti-CD11c-fluorescein isothiocyanate (FITC) (clone N418), anti-F4-80-PE-Cy7 (clone BM8), and anti-T cell receptor β (TCRβ)-allophycocyanin (clone H57-597) were obtained from BD Biosciences, San Jose, CA. Murine lungs were collected at 48 h postinfection, and excised lung tissue was digested with type II collagenase and DNase (1 mg/ml and 50 U/ml, respectively; Worthington, Lakewood, NJ) to obtain a single-cell suspension, as described previously (101). For collection of BALF, mice were sacrificed at 48 h postinfection and lungs were washed twice with 1 ml of PBS via a cannula. Cells in the pulmonary single-cell suspension or in the BALF were stained with relevant monoclonal antibodies (MAbs) on ice, washed, and run through a FACSCalibur flow cytometer (BD Biosciences). Fluorescence intensities of the stained cells were determined. Data were analyzed using FlowJo software (Tree Star) to determine the numbers of PMNs (Ly6G$^+$), dendritic cells (F4/80$^-$ CD11c$^+$), macrophages (F4/80$^+$), and T cells (TCRβ^+) in both the lung single-cell suspensions and BALF.

Statistical analyses. Statistical analysis was performed using the GraphPad Prism program (GraphPad Software). The log rank (Mantel-Cox) test was performed for survival curve analysis. All quantitative results were analyzed by one-way analysis of variance (ANOVA). In cases where data sets contained more than two groups, one-way ANOVA was followed by individual Student's *t* test analyses. *P* values of <0.05 were considered significant in all cases.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00280-17.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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