Hemolytic Phospholipase C Inhibition Protects Lung Function during *Pseudomonas aeruginosa* Infection

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***Rationale*: The opportunistic pathogen *Pseudomonas aeruginosa* causes both acute and chronic lung infections and is particularly problematic in patients with cystic fibrosis and those undergoing mechanical ventilation. Decreased lung function contributes significantly to morbidity and mortality during *P. aeruginosa* infection, and damage inflicted by *P. aeruginosa* virulence factors contributes to lung function decline.**

***Objectives*: We sought to describe direct contribution of a bacterial phospholipase C/sphingomyelinase, PlcHR, to alteration of host lung physiology and characterize a potential therapeutic for protection of lung function.**

***Methods*: We infected C57Bl/6 mice with *P. aeruginosa* wild-type or isogenic *plcHR* deletion strains and measured lung function using computer-controlled ventilators. For *in vivo* testing, miltefosine was delivered intraperitoneally 1 hour after infection. Infection and respiratory endpoints were at 24 hours after infection.**

***Measurements and Main Results*: *P. aeruginosa* wild-type infection caused significant lung function impairment, whereas the effects of a Δ*plcHR* strain infection were much less severe. Surfactometry analysis of bronchoalveolar lavage fluid indicated that PlcHR decreased pulmonary surfactant function. Miltefosine has structural similarity to the PC and sphingomyelin substrates of PlcHR, and we found that it inhibits the cleavage of these choline-containing lipids *in vitro*. Miltefosine administration after *P. aeruginosa* infection limited the negative effects of PlcHR activity on lung function.**

***Conclusions*: We have directly linked production of a single virulence factor in *P. aeruginosa* with effects on lung function, and demonstrated that the inhibitor miltefosine protects lung function from PlcHR-dependent surfactant dysfunction.**

Keywords: *Pseudomonas*; surfactant; phospholipase; respiratory mechanics

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Although studies have examined the role of many *Pseudomonas aeruginosa* virulence factors during infection, none have demonstrated direct effects on pulmonary physiology, which manifests because there are no therapies designed to explicitly protect lung function during bacterial infection.

What This Study Adds to the Field

Here we show that a single extracellular bacterial protein with phospholipase C/sphingomyelinase (PC-PLC/SMase) activity alters respiratory physiology during infection and we have identified a small molecule that inhibits this PC-PLC/SMase activity and demonstrated that it can protect lung function during infection in the mouse. Conservation of this specific PC-PLC/SMase family in other respiratory pathogens, including *Mycobacterium tuberculosis*, suggests that this inhibitor could have broad efficacy.

Pseudomonas aeruginosa is a common, gram-negative opportunistic pathogen responsible for 8–16% of nosocomial infections (1). Chronic *P. aeruginosa* infections are common in the lungs of patients with cystic fibrosis (CF) and are associated with over 80% of the morbidity and mortality in this population (2–4). Chronic *P. aeruginosa* infections are also seen in patients undergoing mechanical ventilation, and in people with chronic obstructive pulmonary disease (5, 6). Acute *P. aeruginosa* pneumonia is associated with high mortality (7, 8).

Numerous *P. aeruginosa* virulence factors impact the growth, survival, and immune evasion of *P. aeruginosa in vivo*, and contribute to host inflammation and tissue damage during *P. aeruginosa* lung infections (9). One secreted virulence factor in particular, PlcHR, has been linked to declines in lung function and increase in frequency of exacerbation in patients with CF (10, 11). The preferred substrates for PlcH activity are phosphatidylcholine (PC) and sphingomyelin (12), lipids that make up the bulk of cellular membranes and pulmonary surfactant. Many reports detail the potential detrimental effects of PlcH PC-phospholipase C/sphingomyelinase (PC-PLC/SMase) activity during infection (13), but have not examined pulmonary function.

PlcH may influence patient status and disease course through its effects on pulmonary surfactant, a fluid largely composed of dipalmitoylphosphatidylcholine (14). *P. aeruginosa* phospholipases from culture supernatants are sufficient for surfactant PC degradation (15), and we have shown that *P. aeruginosa* PlcH is necessary for the efficient breakdown of lung surfactant PC (16). Pulmonary surfactant lowers the surface tension of the

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airway–surface liquid and surfactant dysfunction has been observed in patients with CF with chronic *P. aeruginosa* infections (17–19) and in individuals with *P. aeruginosa* and other bacterial pneumonias (20, 21). Decreased surfactant activity has been proposed to be a contributing factor to lung disease in CF (22) and other respiratory illnesses including pneumonia (20, 23) and chronic bacterial infections (24). Lung surfactant homeostasis in disease is complex, and multiple host and pathogen factors have the potential to alter the production, function, or levels of lung surfactant components during *P. aeruginosa* infections (25–29).

Given the potential for contribution of PlcHR to pulmonary surfactant function, we hypothesized that isogenic *plcHR* mutants would cause less damage to the pulmonary surfactant system and therefore result in improved lung function. Currently, there are no therapies directed exclusively at protection of surfactant function during infection. We predicted that a small molecule inhibitor of PlcHR would be a promising therapeutic agent during *P. aeruginosa* lung infections directed at protection of respiratory function. Some of the results of these studies have been previously reported in the form of abstracts (30, 31).

METHODS

Strains, Growth Conditions, and Strain Construction

P. aeruginosa strain PAO1 and the isogenic Δ*plcHR* deletion strain, and *Escherichia coli* strains, were maintained on Lysogeny Broth medium. When necessary, gentamicin was added to 10 μg/ml for *E. coli*, and 50 μg/ml for *P. aeruginosa*. The *plcHR* deletion in our PAO1 strain was made via the pMQ30 plasmid by recombination as described previously (32, 33) using the following primers: plcH-GOI-F 5′-CGACGATACTGTCCCAACCT-3′, plcH-SOE-R 5′catgatcggcgatGGTACCcagctcctcgtcggCAAGCGTCACCAAAACTTCA-3′, plcH-SOE-F 5′-ccgacgaggagctgGGTACCatcgccgatcatgGTTTTATTTCCCGGGTGGTT-3′, and plcH-GOI-R 5′-CAGCAGTTGTTCGTCGACAT-3′. Similar to results we reported previously with a similar deletion mutant, the Δ*plcHR* deletion produced no choline-induced nitrophenolphosporylcholine (NPPC) hydrolysis activity (16).

Sputum RNA Isolation and Quantitative Reverse-Transcriptase Polymerase Chain Reaction

Sputum samples from patients with CF over the age of 12 with a history of infection by *P. aeruginosa* were collected in accordance with the ethical guidelines of the Committee for the Protection of Human Subjects at Dartmouth College (Hanover, NH). Immediately after collection, two volumes of RNA-protect bacterial agent (Qiagen, Valencia, CA) were added to maintain RNA integrity and the samples were kept at −80°C until analysis. On the day of RNA isolation, samples were thawed and liquefied with progressively smaller needles for processing, 40–80 μl of 2% dithiothreitol was added, and the sample was then incubated on ice for 5 minutes and spun down at 13,000 × rpm for 3 minutes.

RNA extraction was done via a phenol chloroform extraction protocol adapted from Mamessier and coworkers (34) with minor modifications as described next. The recovered RNA was treated with RQ1 DNase (Promega, Madison, WI) to remove contaminating DNA, and DNases were removed by RNeasy kit (Qiagen). cDNA synthesis was accomplished using Superscript III (Invitrogen) and 5′-NSNSNSNSNS-3′ primers in a 20-μl reaction. Quantitative analysis of *plcH* and *ppiD* levels was determined using Taqman duplex polymerase chain reaction to find the ratios of *plcH/ppiD*. Primers and probes used for *plcH* expression were as follows: forward primer 5′-GGCTCCAGGAGCAGAACAAC-3′; reverse primer 5′-TGCCAGAGGTCCGAATCG-3′; probe 5′-6FAM-AACGCCCTCGCCTGGTTCAGGA-MGBNFQ-3′. Primers and probes used for *ppiD* expression were as follows: forward primer 5′-CGGGCACCGGTTTCG-3′; reverse primer 5′-AAGTCGCGGGTCTGCTTCT-3′; probe 5′-VIC-CACCGACAACGAATTGCAGTCCTTCG-MGBNFQ-3′.

As controls, ratios of *plcH/ppiD* were also obtained from wild-type (WT) *P. aeruginosa* grown in media in which *plcH* mRNA levels were induced or at basal levels. The induced cultures were grown in 3-(N-morpholino)propanesulfonic acid medium with 2-mM sodium pyruvate, 8-μM ferric chloride, and 3% (vol/vol) bovine lung surfactant (Survanta), whereas uninduced cells had 20-mM pyruvate and no surfactant.

Purification of PlcHR

Purifications of active PlcHR and the catalytic mutant PlcHR T178A were done according to the methods of Vasil and colleagues and was determined to be LPS-free (35, 36).

Hemolysis and NPPC Hydrolysis Assays

Purified PlcHR was diluted to 10 μg/ml in phosphate-buffered saline (PBS) and miltefosine (Sigma) was added to generate the specific final concentrations. Washed sheep erythrocytes were added to the hemolysis reaction and incubated at 37°C. Intact erythrocytes were pelleted and hemoglobin in the supernatants was measured by absorbance at 540 nm. Hemolysis was reported as percent hemolysis with the hemolysis caused by PlcHR in the absence of miltefosine set at 100%. PLC activity measurement by NPPC hydrolysis was conducted as previously reported by our group (16).

Mouse Lung Infection

The protocol for animal infection and respiratory physiology measurements was approved by the Institutional Animal Care and Use Committee of the University of Vermont (Burlington, VT), in accordance with Association for Assessment and Accreditation of Laboratory Animal Care guidelines. All surgeries were performed under pentobarbital anesthesia, ventilated paralyzed animals were monitored for distress using ECG, and all efforts were made to minimize animal suffering.

Overnight LB cultures of *P. aeruginosa* were measured by OD_{600}, pelleted, washed twice with PBS, and resuspended to give 5×10^7 viable *P. aeruginosa* in 40 μl. Actual inoculum was determined by serial dilution of the input bacterial suspension on *Pseudomonas* isolation agar (Difco). Adult male C57Bl/6J mice, 8–12 weeks old (Jackson Laboratories, Detroit, MI), were inoculated with 40 μl (5×10^7 CFU) of *P. aeruginosa* PAO1 or isogenic Δ*plcHR* via oropharyngeal aspiration following brief anesthesia with isoflourane (16, 37). When appropriate, miltefosine was delivered via intraperitoneal injection 1 hour after infection at a total dose of 10 mg/kg. At 24 hours after infection, mice were anesthetized with intraperitoneal sodium pentobarbital, tracheas were cannulated, and bronchoalveolar lavage fluid (BALF) collected. Lungs and spleens were excised and immediately placed into 1 ml of cold PBS followed by homogenization. Viable bacterial counts in lungs and spleens were determined by plating serial dilutions of organ homogenate onto *Pseudomonas* isolation agar plates followed by incubation at 37°C for 24 hours. White blood cell counts in the BALF were done using an Advia automated cell counter (Siemens, Berlin, Germany) and cell type was done by manual examination of hematoxylin and eosin–stained slides. To measure lung weights, excised lungs were blotted three times and weighed in plastic weigh boats. For histology lungs were inflation-fixed at 20 cm H_2O pressure with buffered formalin for 24 hours, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Cell-free protein content in the BALF was determined by Bradford assay with bovine serum albumin as the standard.

Mouse Ventilation and Lung Physiology

For mouse ventilation studies, mice were anesthetized with pentobarbital; tracheas were cannulated; they were placed on the ventilator and paralyzed with intraperitoneal pancuronium bromide (0.5 mg/kg); and heart rate was monitored by ECG to ensure proper anesthesia. Ventilation, pressure–volume (P-V) measurements, and respiratory impedance were conducted as previously described (38–40). A full description of the ventilation techniques and analysis can be found in the online supplement.

For direct instillation of purified PlcHR via the cannula, the dose of 50 ng PlcHR per mouse was based on the intranasal instillation used by Wieland and colleagues (41), which yields a high but nonlethal dose. PlcHR caused dose-dependent effects on lung function (data not shown); the dose chosen caused surfactant dysfunction at 1.5 hours

after instillation, which is comparable with that observed after infection with WT *P. aeruginosa* for 24 hours.

Surfactant Activity

For surfactant analysis, lavage was conducted from mice with sterile normal saline. Lavage was fractionated (22), extracted, and inorganic phosphate content of the lipid fraction was measured (42). The large aggregate fractions were brought to 1 mg/ml phospholipid concentration in capillary surfactant buffer and analyzed using a capillary surfactometer (Calmia Biomedical, Toronto, ON, Canada) according to Lema and colleagues (15). Each sample (from one mouse) was measured on the capillary surfactometer six times, and the average from each mouse ($n > 6$ per group) was averaged to generate the mean reported in the results section.

For *in vitro* surfactometry with PlcHR, samples of Survanta were diluted to 2 mg/ml phospholipid in capillary surfactant buffer, to which miltefosine and purified PlcHR in capillary surfactant buffer were added. Additions of miltefosine and PlcHR to subsequent samples were staged 4 to 5 minutes apart to account for the 120-second surfactometer run, and the loading and placement of the capillary for the next sample.

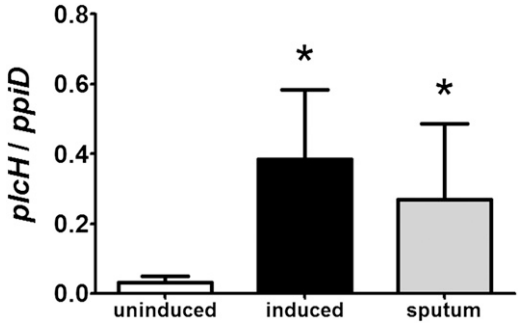

Figure 1. Real-time quantification of relative *plcH* mRNA levels in *Pseudomonas aeruginosa* RNA isolated from cystic fibrosis sputum. The *plcH/ppiD* mRNA ratios from uninduced (pyruvate-grown) and induced (surfactant-grown) *P. aeruginosa* were determined in 4-hour cultures from four independent experiments and averaged together. Ratios of *plcH* to *ppiD* in sputum samples represent the average from nine patients. *Error bars* represent the SD. $*P < 0.05$.

RESULTS

plcH mRNA Levels in CF Sputum

In the mouse lung, we found that induction of *plcH* mRNA levels is dependent on the GbdR transcription factor (16), which is activated by glycine betaine and dimethylglycine, two catabolic intermediates in the pathway for the catabolism of choline-containing lipids (33). Similar regulation of *plcH* transcription was also observed in lung surfactant (33). Using the ratio of *plcH* to *ppiD*, a control transcript that remains at constant levels in all conditions tested to indicate levels of *plcH* expression, we determined if *plcH* is also highly expressed in the sputum of patients with CF who are chronically infected with *P. aeruginosa*. Total RNA from the sputum of nine patients with CF infected with *P. aeruginosa* was isolated, and *plcH* and *ppiD* mRNA levels were measured by quantitative Taqman reverse-transcriptase polymerase chain reaction. For comparison, total *P. aeruginosa* RNA was extracted from multiple independent cultures grown on different days in a defined medium with pyruvate as the sole source of carbon, in which no PlcH activity is detected, or in medium with PC-rich lung surfactant, which induces *plcH* expression in a GbdR-dependent manner (16). There was a 12-fold increase in *plcH* expression in the induced controls compared with the uninduced controls ($P = 0.01$) (Figure 1). The high ratio of *plcH/ppiD* levels seen in the sputum samples compared with the uninduced controls is similar to the induced controls indicating expression of *plcH* in CF sputum ($P = 0.01$). These results suggest that *plcH* is expressed in chronic CF infections, and this finding is consistent with a previous study by Hollsing and coworkers (43) that showed detection of serum antibodies to PlcH in all 62 of the patients with CF infected with *P. aeruginosa* analyzed.

Purified PlcHR Directly Affects Lung Function

Because of correlations between decreased lung function and poor prognosis in patients infected with *P. aeruginosa* (44), and the evidence that *plcH* is robustly expressed during chronic infections (Figure 1), we explored the effects of PlcH specifically on lung function using purified PlcH protein. PlcH is purified from culture supernatants in complex with PlcR (35), and thus PlcHR was used in these experiments. PlcHR (50 ng as described in the METHODS section) was administered intratracheally to anesthetized mice on a ventilator, and lung function was assessed by measuring the P-V relationship and impedance using forced oscillations on ventilated mice.

Single P-V loops are comprised of an inspiratory limb and an expiratory limb. The curves shown represent the average of data from multiple animals measured at the same time point after treatment. Here we have focused our attention on the inspiratory limb in the range of normal ventilation because this region of the P-V curve is most sensitive to changes in surfactant function (45). Buffer alone (PBS) did not affect lung function as shown by the similarities of the inspiratory curve, which is always the right limb of the P-V loop, and the expiratory curve. Buffer-treated control mice had P-V loops similar to those of untreated mice (data not shown). PlcHR instillation into the lung led to a striking increase in hysteresis or widening between the two arms of the P-V loop showing perturbation of the inspiratory phase (Figure 2A). To determine if the effects of PlcHR on lung function were dependent on catalytic activity, equivalent amounts of catalytically inactive PlcH variant (T178A) (35) were added as PlcH(T178A)R, and lung function was measured. Catalytically inactive PlcH(T178A)R did not lead to P-V loop changes relative to the buffer control (Figure 2A).

The P-V loop profile observed on instillation of PlcHR can be indicative of decreased activity of the pulmonary surfactant, which is largely composed of PC lipids that are known substrates of PlcHR (46). The shape of the P-V curve was indicative of surfactant dysfunction. In humans, compliance (C, L/cm H_2O) measurements are often used to assess surfactant function. Because of the differences between humans and mice, particularly in the methods used to quantify lung function, we have chosen to report quasistatic elastance (E) which is the mathematical inverse of compliance. E, and by direct relation C, is the physiologically most relevant metric influenced by pulmonary surfactant. E is measured from the slope of the P-V inspiratory limb, which is increased in the setting of surfactant dysfunction and distal lung closure (27, 47). Because we calculated E from the range of points defining the middle to end of tidal volume, our reported E emphasizes any pathology altering lung physiology in the range of normal breathing. Treatment with catalytically active PlcHR caused a large increase in E (Figure 2B), supporting a direct effect on surfactant function.

The parameter E, as measured previously, is a largely time-independent metric because it is measured after a 1-second pause in volume adjustment to allow for tissue rheology effects to dampen. An important missing component of E, therefore, is the time dependence of the P-V relationship. The parameter H, termed here "lung stiffness/elastance," is a time-resolved metric derived from measures of impedance fitted to the constant phase

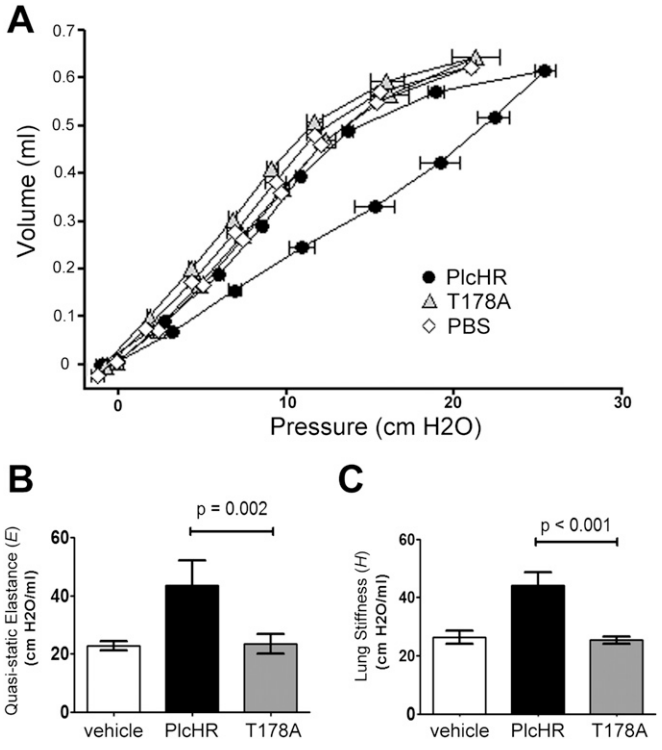

Figure 2. Intratracheal instillation of purified PlcHR resulted in a loss of lung function. (*A*) Pressure–volume (P-V) loops were measured in vehicle-treated (phosphate-buffered saline [PBS], *white diamonds*), PlcHR-treated (*black circles*), and catalytically inactive PlcHR T178A-treated (*gray triangles*) animals. Both PlcHR additions were 50 ng of protein in 50 μl of PBS. The quasistatic pressure is plotted against volume and the symbols represent mean ± SEM. Y-error bars have been removed for clarity. (*B*) Quasistatic elastance (*E*) calculated from the inspiratory limb of the P-V curves in (*A*). (*C*) Lung stiffness (*H*) calculated from respiratory impedance measurements based on the constant phase model. Means in (*B*) and (*C*) are shown ± 1 SD from a representative experiment of five mice per group. Two independent experiments with five animals per infected group showed similar results. Tests of significance conducted using a one-way analysis of variance with a Bonferroni multiple comparison test.

model and is a sensitive measure of distal lung closure (40, 48, 49). Thus, *E* and *H* are complimentary measurements of the relationship between pressure and volume in the lung. The nearly twofold increase in *H* on instillation of active PlcHR, but not the catalytically inactive variant (Figure 2C), indicated significant changes in distal airway closure to levels that were comparable with those observed in mouse models of acute asthma or lung injury (39, 48). Dysfunction of pulmonary surfactant and resultant airway closure is the physiologic phenomenon that best explains the correspondence of data from *E*, *H*, and P-V curve shape.

The Role of PlcHR in *P. aeruginosa* Alteration of Mouse Lung Function

To determine the role of PlcHR in *P. aeruginosa* mouse lung infections, a low-dose inoculum (1×10^6 CFU) of WT and Δ*plcHR P. aeruginosa* PAO1 was administered into the lungs of mice by oropharyngeal aspiration. PlcHR was found to be important for colonization because the WT was present at approximately 40-fold greater levels than the isogenic Δ*plcHR* strain after 24 hours (*see* Figure E1 in the online supplement). The defects in virulence in mutants lacking *plcH* were consistent data for other mouse models of infection (50, 51). To specifically examine the effects of PlcHR on lung function during *P. aeruginosa* infection of the mouse lung, we developed a lung infection regime in which WT and Δ*plcHR* strains were present in the lung at equivalent CFU to avoid differences in bacterial load or inflammation, which could indirectly affect lung physiology. We found that animals infected with either WT or the Δ*plcHR* mutant, at a dose of 5×10^7 CFU per mouse, had no detectable differences in bacterial burden or inflammation at either 6 (*see* Figure E2) or 24 hours (Figure 3A). In addition, levels of infiltrating white blood cells and lung histology were similar in WT and Δ*plcHR*-infected mice (Figures 3B and 3C), suggesting that there were no significant differences in airway inflammation. Furthermore, cell-free protein as measured in the BALF showed no difference in protein content of the BALF between the two infections (1.11 ± 0.18 mg/ml for WT infection compared with 1.06 ± 0.23 mg/ml in Δ*plcHR* infection; $P = 0.68$) suggesting there were no detectable differences in epithelial barrier function at this time point. At 24 hours, both *P. aeruginosa* WT and Δ*plcHR* caused systemic bacteremia, and there were no significant differences in spleen CFU (Figure 3A) or blood bacterial burden (not shown) between strains.

On qualitative examination, the mice infected with Δ*plcHR* seemed to have less respiratory distress than mice infected with WT PAO1. This observation suggested that some aspect of respiratory physiology was different between the two groups. Based on the data that showed that purified PlcHR induced lung closure *in vivo* (Figure 2), we predicted that a Δ*plcHR* mutant would have a decreased impact on lung mechanics during infection compared with the WT. P-V and impedance measurements were performed as described previously on mice

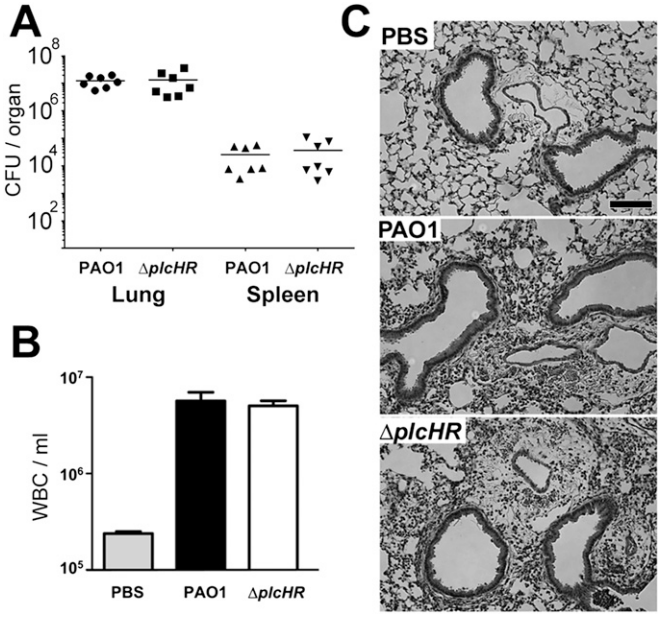

Figure 3. Deletion of *plcHR* did not alter bacterial load or inflammation at high infective dose. (*A*) Bacterial burden from whole homogenized lung and spleen. CFU counts from individual mice plotted with mean noted by the *horizontal lines*. Means are not significantly different. (*B*) WBC infiltration into the BALF as measured by automated counter (Advia). Mean ± SEM plotted for 7 mice/group, and were not significantly different. (*C*) Hematoxylin and eosin stained lung sections from control and infected animals. *Scale bar* = 100 μm. Histology is from a representative experiment with 4 mice per group and is representative of two independent experiments. Statistical significance for bacterial burden calculated using a two-tailed *t* test.

infected with either WT *P. aeruginosa* PAO1 or the isogenic Δ*plcHR* strain, both at the higher dose. WT infected animals showed extremely pathologic lung physiology as assessed by hysteresis of the P-V curve and decrease in the slope of the inspiratory limb (Figures 4A and 4B). Infection with the Δ*plcHR* strain resulted in lung physiology that is abnormal compared with mock-infected mice (PBS instillation), but resulted in better lung function compared with animals infected with WT *P. aeruginosa*, using measures of P-V hysteresis (Figure 4A), *E* (Figure 4B), and *H* (Figure 4C). Based on these data, for the same amount of breathing effort, the Δ*plcHR*-infected mice were able to open approximately twice as much lung for gas exchange.

To verify that this difference in lung function was caused by the loss of the *plcHR* genes and not an unlinked mutation, we used two methods of genetic complementation. Both of these methods show alterations in lung function proportional to the complementation of PlcH enzymatic activity (*see* Figure E3). Another possibility was that extravascular fluid accumulation was different in the WT versus Δ*plcHR* infections, because of the known lethal effect of PlcHR on endothelial cells and on angiogenesis (35), and that this resulted in altered lung function. To test this hypothesis we measured wet lung weights from mice who were uninfected, infected with PAO1, and infected with Δ*plcHR* and saw no statistically significant difference in wet weights between animals infected with PAO1 and Δ*plcHR* ($P > 0.10$). For mice who were uninfected, infected with PAO1, and infected with Δ*plcHR*, the wet weights of the excised lungs were 0.198 ± 0.009, 0.272 ± 0.006, and 0.259 ± 0.007, respectively.

Direct Measurement of Lung Surfactant Activity from WT Mice and Mice Infected with Δ*plcHR*

The lung physiology measurements from our instillation and infection experiments suggested that PlcHR could alter pulmonary surfactant function *in vivo*. To test this hypothesis, we analyzed surfactant recovered from lung lavage of WT mice or mice infected with Δ*plcHR* 24 hours after infection. Capillary surfactometry measures the ability of surfactant to maintain an open passage through a glass capillary that compares to the size of a respiratory bronchiole (0.22 mm). Using this technique, surfactant function is reported as the percent of time the capillary remains open, with a percentage open time of 100% representing fully functional surfactant, and an open time of 0% representing completely nonfunctional surfactant. To measure surfactant function, we prepared the large aggregate (LA) fraction of pulmonary surfactant from lavage samples and directly measured surfactant function using a capillary surfactometer. Our LA preparations from healthy mice show slightly more than 80% open time (Figure 4D). The WT infected mice showed compromised surfactant function (~ 40% open time), but infection with Δ*plcHR* led to much less of an effect on pulmonary surfactant function (~ 60% open time) compared with WT infection ($P = 0.0105$) (Figure 4D). Surfactant function can be influenced by total surfactant phospholipid levels, the distribution between small aggregate (SA) and large aggregate (LA) fractions, or levels of particular phospholipids. We determined that neither the total lavaged phospholipid content nor the large aggregate (LA) to small aggregate (SA) ratio was different when comparing surfactant from Δ*plcHR* with WT mice infected with *P. aeruginosa* (data not shown). Although it is not surprising that there was still some perturbation of surfactant function in the animals infected with Δ*plcHR*, these data indicated that the preservation of pulmonary function in mice infected with Δ*plcHR* was predominately caused by direct alteration of surfactant function. These data are consistent with the findings of Lema and coworkers (15), which showed that

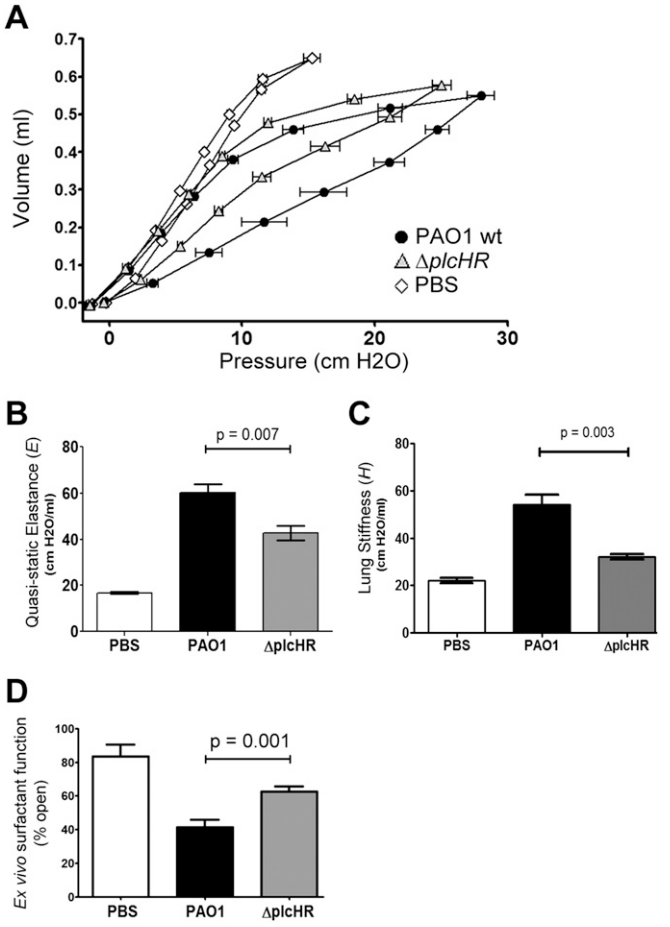

Figure 4. Deletion of *plcHR* alters respiratory mechanics during infection. (*A*) Pressure–volume (P-V) loops measured in sham-treated (*white diamonds*) and *Pseudomonas aeruginosa*–infected mice (*black circles* for wild-type PAO1 infected; *gray triangles* for Δ*plcHR* infected). The quasi-static pressure is plotted against volume and the symbols represent mean ± SEM. Y-error bars have been removed for clarity. (*B*) Quasi-static elastance (*E*) calculated from the inspiratory limb of the P-V curves in (*D*). (C) Lung stiffness (*H*) calculated from respiratory impedance measurements based on the constant phase model. (*D*) Capillary surfactometry was used to assess the surfactant function of the large aggregate fraction of mouse bronchoalveolar lavage fluid. Higher values of percent open indicate better surfactant function. The *black bars* and *gray bars* represent surfactant function from mice infected by wild-type and Δ*plcHR P. aeruginosa*, respectively. Data for *A*–*C* are from the same representative experiment with seven mice per group in the infected groups and four mice per group in the control (phosphate-buffered saline [PBS]) group. Data are representative of two independent experiments. Data for *D* are from one representative experiment, where three independent experiments with at least five animals per infected group show similar results. Mean plotted ± SEM for a minimum of six mice as described in the METHODS section. Tests of significance conducted using a one-way analysis of variance with a Bonferroni multiple comparison post-test.

P. aeruginosa supernatants directly decrease the PC fraction of lung surfactant.

Miltefosine Inhibits PlcHR Activity and PlcHR Disruption of Surfactant Function *In Vitro*

In search of potential inhibitors of PlcH, we tested whether the ether-linked phosphocholine compound, miltefosine (hexadecylphosphocholine), which is already in use clinically in the

treatment of leishmaniasis (52, 53), could block PlcH activity toward the colorimetric substrate, NPPC. Miltefosine effectively inhibited NPPC cleavage at 1.5 μM when purified PlcHR was used (Figure 5A). PlcHR is also capable of hemolysis of sheep erythrocytes because of its ability to cleave sphingomyelin (54, 55), which is the only choline phospholipid in the outer leaflet of sheep erythrocytes. Miltefosine prevented hemolysis by 10 μg/ml PlcHR at concentrations down to 250 nM (Figure 5B).

Because we had linked loss of PlcH expression to surfactant dysfunction *in vivo*, we measured the ability of miltefosine to protect a bovine surfactant preparation (Survanta) from PlcHR-dependent degradation and loss of function *in vitro* using capillary surfactometry. As shown in Figure 5C, miltefosine was able to protect surfactant function from the disruptive activity of 10 μg/ml PlcHR *in vitro* in a dose-dependent manner at concentrations down to 250 nM. Miltefosine alone did not alter surfactant activity (Figure 5C).

Miltefosine Protects Lung Function in a Mouse Model of *P. aeruginosa* Pneumonia

Based on our *in vitro* inhibition results, we hypothesized that miltefosine might provide therapeutic benefit in our mouse lung infection model by inhibiting PlcH activity. To test this hypothesis, we infected mice with WT PAO1 *P. aeruginosa* via oropharyngeal aspiration as described previously. One hour after infection, either miltefosine (10 mg/kg) or vehicle control (PBS) was injected intraperitoneally. Lung function was assessed at 24 hours after infection. We made the qualitative observation that the mice treated with miltefosine were in less respiratory distress than their untreated counterparts, similar to observations with the mice infected with Δ*plcHR* relative to mice infected with WT animals. We therefore examined the lung physiology of miltefosine-treated versus mock-treated animals.

Treatment with miltefosine resulted in significant protection of lung function as evidenced by measures of P-V, *E*, and *H* in the setting of infection (Figures 6A–6C). Importantly, this dramatic protection of lung function was apparent in the absence of any measureable changes in bacterial burden or gross inflammation (*see* Figure E4). Injection of miltefosine or PBS into uninfected mice had no detectable effect on lung physiology (Figures 6B and 6C, fourth bar in each panel).

To determine if miltefosine functioned primarily through inhibition of PlcHR-mediated surfactant dysfunction *in vivo*, miltefosine effects on lung physiology in mice infected with a Δ*plcHR* strain was measured. In support of PlcH inhibition being the primary mechanism governing miltefosine's beneficial effects, miltefosine provided no protection of lung function in mice infected with a Δ*plcHR* strain of *P. aeruginosa* (Figures 6B and 6C, compare third and sixth bars in each panel).

Based on our hypothesis that miltefosine functions via inhibition of PlcHR enzyme activity, we predicted that miltefosine treatment should protect surfactant function during infection. We demonstrated that treatment of infected mice with miltefosine results in improved surfactant function compared with the vehicle control ($P = 0.0402$) (Figure 6D). The level of protection of the surfactant system is comparable with that seen when comparing WT with Δ*plcHR* infection (Figure 4D).

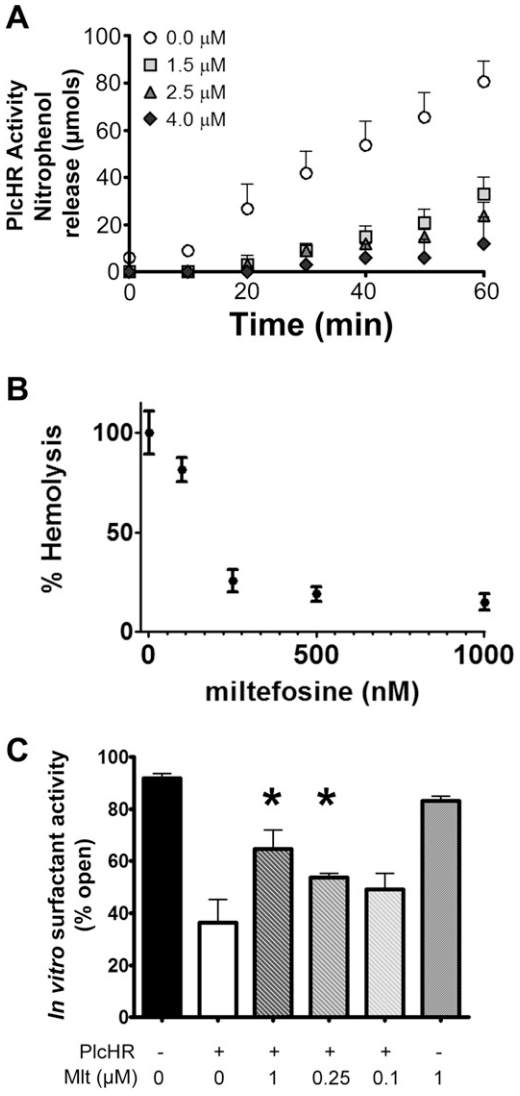

Figure 5. Miltefosine inhibited PlcHR hemolysis, enzymatic activity, and surfactant dysfunction *in vitro*. (*A*) Miltefosine inhibited nitrophenolphosporylcholine hydrolysis by purified PlcHR, measured by nitrophenol release followed as a change in absorbance at 410 nm. The legend indicates the concentration of miltefosine in the reaction (micromolar). Means for parts *A* and *B* are from three replicates and correspond to at least three independent experiments conducted on different days. Error bars indicate SD. (*B*) Miltefosine inhibited PlcHR-dependent hemolysis of sheep erythrocytes in a concentration-dependent manner. (*C*) Capillary surfactometry was used to assess the surfactant function of purified bovine surfactant (Survanta) after treatment with 10 mg/ml PlcHR with or without the labeled doses of miltefosine. Higher values of percent open indicate better surfactant function. Mean plotted ± SEM for six samples as described in the METHODS section. Data are representative of two independent experiments and significance conducted using a one-way analysis of variance with a Bonferroni multiple comparison test. $*P < 0.05$.

DISCUSSION

We have demonstrated that PlcH, together with its nonenzymatic chaperone PlcR, causes dysfunction of mammalian pulmonary surfactant during an experimental model of *P. aeruginosa* lung infection. Treatment of infected mice with injected miltefosine was able to abrogate PlcH-dependent surfactant dysfunction to a degree similar to that seen in infections with a Δ*plcHR* mutant. The impact of miltefosine protection from PlcHR activity was best demonstrated by the failure of miltefosine to provide physiologic benefit to mice infected with a Δ*plcHR* strain (Figures 6B and 6C). Direct measurement of *ex vivo*

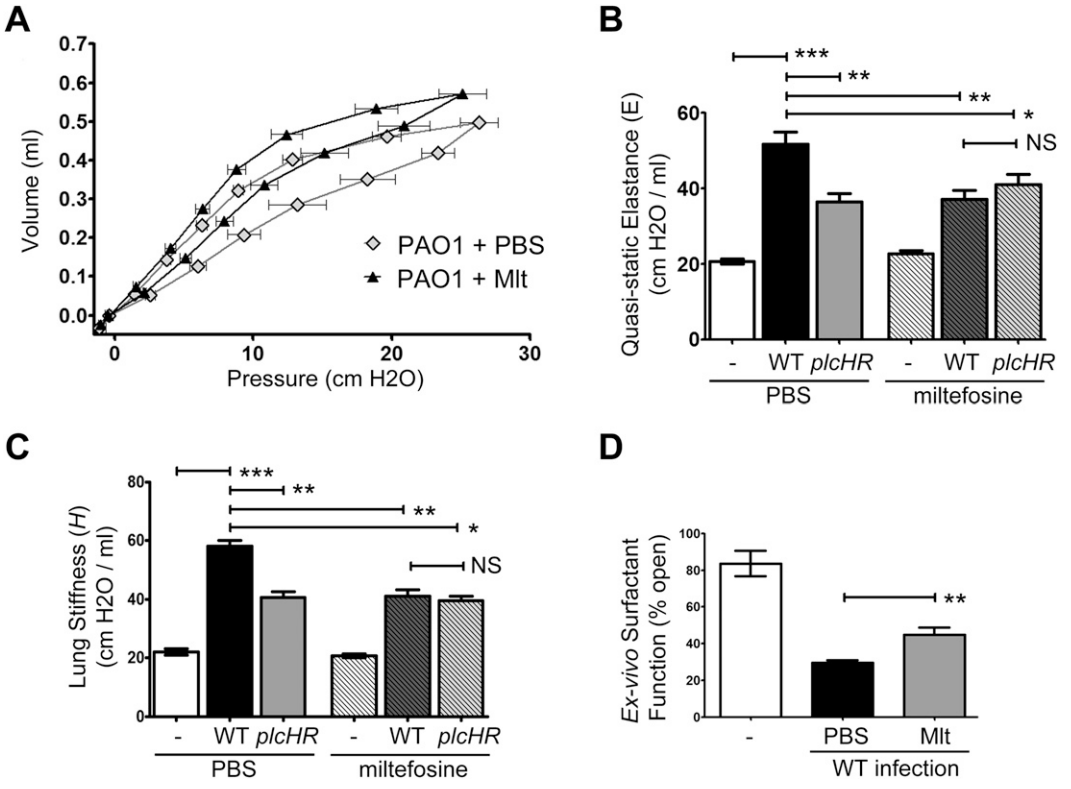

Figure 6. Miltefosine protects against PlcHR-dependent alteration in lung physiology during *Pseudomonas aeruginosa* infection. (*A*) Pressure–volume (P-V) loops measured in mice infected for 24 hours with PAO1 that received injection 1 hour after infection of phosphate-buffered saline (PBS) (vehicle, *gray diamonds*) or 10 mg/kg miltefosine (*black triangles*). The quasistatic pressure is plotted against volume and the symbols represent mean ± SEM for six mice per group. Y-error bars have been removed for clarity. (*B*) Quasistatic elastance (*E*) calculated from the inspiratory limb of the P-V curves in (*A*). (*C*) Lung stiffness (*H*) calculated from respiratory impedance measurements based on the constant phase model. Means in (*B*) and (*C*) are shown ± 1 SD from a representative experiment of six mice per group for PAO1-infected mice, and five mice per group for Δ*plcHR* infected mice. (*D*) Capillary surfactometry was used to assess the surfactant function of the large aggregate fraction of mouse bronchoalveolar lavage fluid. Higher values of percent open indicate better surfactant function. Bars represent surfactant function from mice infected with wild-type (WT) *P. aeruginosa* that received injection of PBS (*black*) or 10 mg/kg miltefosine (*gray*). Mean plotted ± SEM for a minimum of six mice as described in the METHODS section. Data are representative of two independent experiments. For all figure parts, three independent experiments with at least five animals per infected group show similar results. Test for significance done using one-way analysis of variance with a Bonferroni multiple comparison post-test (*B–D*). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

surfactant function from infected animals also demonstrated that much of the protection of pulmonary function could be attributed to preservation of surfactant function by miltefosine (Figures 4 and 6). This study provides the first direct evidence that miltefosine treatment, and PlcHR inhibition in general, may have beneficial effects for patients with *P. aeruginosa* lung infections. Orthologs of PlcHR are present in a number of important lung pathogens including the CF pathogens *Burkholderia cepacia* and *Burkholderia cenocepacia*, and the select agents *Burkholderia pseudomallei* and *Burkholderia mallei* (36). Orthologs are also found in the lung pathogens *Mycobacterium tuberculosis*, *Francisella tularensis*, and *Bordetella pertussis* (36). Interestingly, *M. tuberculosis* strains may carry as many as four distinct genes encoding orthologs of PlcH (36). Thus, miltefosine or a derivative compound may be beneficial in protecting lung function during *P. aeruginosa* infections and during infection with other bacterial pathogens expressing PlcH-like proteins. Further work is needed to address the potential protective effect of miltefosine and inhibition of these homologous virulence determinants during infection with other bacterial pathogens.

We identified miltefosine as an inhibitor of PlcH hemolysis during a directed screen of a small set of PC-like analogs. Miltefosine is currently used as a therapeutic to treat leishmaniasis in several countries (56). The mechanism by which miltefosine protects against leishmania infection is not yet known but some evidence suggests that it affects phosphoplipid metabolism in the parasite (57). Miltefosine has been shown to have some antibacterial activity *in vitro* (58, 59), but *in vivo* efficacy against bacteria has not yet been reported. Our study demonstrates that although miltefosine is not effective as a traditional antibacterial compound *in vivo* (*see* Figure E4), it is capable of dramatically preserving lung function during *P. aeruginosa* infection (Figure 6A). Thus, although we are not decreasing bacterial load or inflammation in the high-dose model of infection, respiratory failure caused by surfactant dysfunction is a life-threatening symptom of bacterial lung infection. The absence of PlcHR or with miltefosine treatment in PlcHR-treated animals results in a 50% increase in the respiratory volume of the lung at pressures generated during tidal breathing. For the same amount of breathing effort, therefore, the mice treated with miltefosine are able to open more lung for gas exchange. This magnitude of change would have profound clinical significance in humans in terms of ventilator settings, time on the ventilator, and oxygenation. The inoculum of *P. aeruginosa* given to these mice is approximately the LD_{50} dose at 48 hours. Given miltefosine's effects we did examine whether *plcHR* deletion or miltefosine treatment improved mouse survival, but neither the deletion nor the treatment altered animal survival (data not shown). This is not particularly surprising because the model leads to severe bacteremia and mice likely die from systemic shock and multiorgan failure rather than respiratory failure.

It is important to note that *P. aeruginosa* possesses activities in addition to those of PlcH that can alter lung surfactant. Specifically in relation to surfactant lipids, Mallampalli and colleagues have shown an alteration of surfactant levels during a chronic infection model with mucoid *P. aeruginosa* that seems to depend on cell–cell contact, but the effector has not been reported (25, 27). ExoU, a phospholipase A_2, also plays a role in *P. aeruginosa* lung infection models (60). ExoU is not

predicted to play a role in surfactant dysfunction because it is a type III secreted effector that is delivered directly from the bacterium to the host cell cytoplasm where it is activated in the presence of mammalian cytoplasmic SOD1 (61). Furthermore, the *P. aeruginosa* strain (i.e., PAO1) used in these studies does not have a gene encoding ExoU. Schmalzer and colleagues (61) have postulated extracellular activation of ExoU by SOD1 in the context of necrotic cells, but this has not been demonstrated to date. Other *P. aeruginosa* phospholipases have not been linked to its virulence in humans or in animal models. Although other virulence determinants likely play important roles during *P. aeruginosa* infection of the lung, this study demonstrates that PlcHR is a major contributor to inactivation of surfactant and subsequent airway closure during *P. aeruginosa* lung infection.

PlcH has many other effects on the host that may be relevant to lung disease in addition to its effects on lung function (Figure 1) and lung colonization in low-dose infections (*see* Figure E1). PlcHR has been shown to alter host innate immune responses in a number of ways (41, 62, 63). Most recently, PlcH has been shown to be selectively proapoptotic to endothelial cells, and to inhibit angiogenesis *in vitro* and *in vivo* (35). Mutants lacking *plcH* produce more localized foci of damage and inflammatory cell congregation suggesting that PlcH production somehow aids in bacterial dispersion in a rat lung chronic infection model (64). PlcH exhibits SMase activity (46), and the product of this activity, ceramide, has been implicated in poor bacterial clearance in CF (65, 66). Bacterial pneumonia is a multifactorial disease and we have noted that addition of purified bovine surfactant or purified mouse large aggregate fractions results in no improvement in our model. This could represent alternate PlcH functions or it may be representative of results from surfactant replacement in human adults with surfactant dysfunction, which rarely demonstrates significant improvement (67). Our data indicate that miltefosine blocks cleavage of both PC-containing surfactant (Figure 5C) and sphingomyelin-rich sheep erythrocytes (Figure 5B) (54, 55), suggesting that miltefosine is an important inhibitor of both PC and sphingomyelin cleavage by PlcH. Future experiments will determine the value of miltefosine, or other PlcH inhibitors yet to be described, in protecting against the other important roles of PlcH during infections of the lung.

In addition to its inhibition of PlcH activity, miltefosine may also have other effects on the host. Although we cannot completely rule out a role for alteration of the host immune system in our current model, we detected no change in immune cell infiltration, immune cell composition, or gross histologic differences (*see* Figure E4, and data not shown). Miltefosine has also been demonstrated to alter lipid metabolism in mammalian cells. Of particular interest for this study, miltefosine can inhibit PC synthesis by inhibiting the proper localization of the cytidylyltransferase (68, 69), and seems to alter the sphingomyelin/ceramide ratio by inhibiting synthesis of sphingomyelin (70, 71). The lipid synthesis effects described previously were seen at higher doses of miltefosine than those required for PlcH inhibition. In our study, a single dose of miltefosine injected intraperitoneally did not result in any measurable alteration of surfactant function in healthy animals (Figures 6B and 6C). This is supported by the *in vitro* inhibition of PlcHR-induced surfactant dysfunction by miltefosine, where effects on *de novo* surfactant synthesis do not play a role (Figure 5).

Our report represents the first *in vivo* demonstration of a role for PlcHR in surfactant dysfunction during a mouse model of pneumonia. These data are consistent with previous reports on the effects of *P. aeruginosa* (15) or commercially available PLC from *Clostridium perfringins* (29) on surfactant activity *in vitro*. Additionally, this is the first *P. aeruginosa* virulence factor that has been directly linked to an alteration of pulmonary physiology unrelated to changes to inflammation and bacterial burden. Thus, PlcHR represents a potential therapeutic target to protect against respiratory failure in patients with acute and chronic *P. aeruginosa* infections. We have identified a PlcHR inhibitor miltefosine that is already being used in humans, and demonstrated its efficacy for protection of mouse lung physiology during *P. aeruginosa* infection. Future clinical studies will examine the impact of miltefosine on lung compliance in the context of bacterial infections. These findings could have substantial translational impact for patients infected with *P. aeruginosa*.

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