Original Paper

Therapeutic Inhaled Sphingosine for Treating Lung Infection in a Mouse Model of Critical Illness

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Abstract

Background/Aims: Sphingosine, a sphingoid long chain base, is a natural lipid with antimicrobial properties. Recent animal studies have shown that preventive sphingosine inhalation can rescue susceptible mice, such as cystic fibrosis-, burn injured- or aged mice from bacterial pulmonary infection. While preventing lung infections in susceptible patients has obvious clinical merit, treatment strategies for an established infection are also direly needed, particularly in the times of rising antibiotic resistance. Here, we tested the potential of sphingosine in treating an established pulmonary infection. ***Methods:*** We used a cecal ligation and puncture (CLP) model in male CF-1 mice and a *Pseudomonas aeruginosa* strain that was isolated from a septic patient (*P. aeruginosa* 762). We determined susceptibility to intranasal infection and ascertained when the pulmonary infection was established by continuous core body temperature monitoring. We quantified sphingosine levels in the tracheal epithelium by immunohistochemistry and studied the effects on sphingosine on bacterial membrane permeabilization and intracellular acidification using fluorescent probes. ***Results:*** We first determined that septic mice are highly susceptible to *P. aeruginosa* infection 2 days after inducing sepsis. Additionally, at this time, sphingosine levels in the tracheal epithelium are significantly reduced as compared to levels in healthy mice. Secondly, upon intranasal *Pseudomonas* inoculation, we ascertained that pulmonary infection was established as early as 2.5 h after inoculation as evidenced by a significant drop in core body temperature. Using these times of infection susceptibility and detection (2 days post CLP, 2.5h after inoculation) we treated with inhaled sphingosine and observed pulmonary bacterial loads reduced to levels found in infected healthy mice after inoculation and decreased infection-associated mortality. Further,

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our data demonstrate that sphingosine induces outer membrane permeabilization, disrupting the membrane potential and leading to intracellular acidification of the bacteria. ***Conclusion:*** Sphingosine shows efficacy in treating *P. aeruginosa* lung infections not only prophylactically, but also therapeutically.

Introduction

Pneumonia is the fourth most common cause of death worldwide and the leading cause of death in low-income countries [1]. Both community-acquired and hospital-acquired bacterial pneumonia are associated with increased morbidity and mortality. The typical treatment for these lung infections is supportive care and antibiotics. Despite this care, a recent study from France described treatment failure rates as high as 31 % [2]. This failure rate is likely due to growing appearance of antibiotic resistant bacteria [3]. Cystic fibrosis and Chronic obstructive pulmonary disease patients, the elderly and critically ill patients are at a particularly high risk for pneumonia. Among the hospitalized group, septic patients, patients who suffered a major trauma (i.e. burn victims) and any patients requiring mechanical ventilation are especially susceptible [4-7].

Sphingosine is a natural lipid and part of the innate defense barrier of the skin and airways [8, 9]. The antimicrobial properties of sphingosine against both gram-positive and gram-negative bacteria were first reported in 1992 [10]. Bacterial killing occurs at low micromolar concentrations [10-13], but the bactericidal mechanism is still unknown. Studies with artificial liposomes and ghost membranes have shown that sphingosine can permeabilize membranes [14, 15]. Additionally, changes in the cell surface of sphingosine-treated bacteria were observed using electron microscopy [12]. The cytoplasmic membrane of the tested Gram-negative bacteria (*Escherichia coli*) appeared intact, whereas the tested Gram-positive bacteria (*Staphylococcus aureus*) showed signs of membrane disruption [12]. Taken together, this indicates that sphingosine may kill Gram-positive bacteria through membrane permeabilization, but how death of Gram-negative bacteria is induced if the inner membrane stays intact remains unclear.

Recently, animal studies have shown that sphingosine inhalation can prevent pulmonary infections with common pathogens such as *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Staphylococcus aureus* (*S. aureus*) [13, 16-19]. No toxic effects of sphingosine inhalation were observed in mice [20] and mini pigs [7]. In these studies, inhaled sphingosine was administered prior to inoculation with bacteria or at very early time-points after inoculation, successfully preventing pneumonia [13, 16-19]. While preventing a lung infection in high-risk patients altogether is certainly an elegant approach, there is still a high demand for effective therapies for established infections. In contrast to these studies, we tested the therapeutic - and not the prophylactic - potential of inhaled sphingosine. We chose a murine model of polymicrobial sepsis - cecal ligation and puncture (CLP) - since septic patients are among the most vulnerable to pneumonia. We first established when after sepsis mice are susceptible to infection. We used a multi-drug resistant clinical *P. aeruginosa* isolate (*P. aeruginosa* 762), as pneumonia caused by *P. aeruginosa* is a major cause of morbidity and mortality in hospitalized patients and antibiotic resistance is a leading cause for antibiotic therapy failure. We confirmed that sphingosine kills this multi-drug resistant strain. We next determined how long it takes for the mice to develop pneumonia after inoculation with *Pseudomonas* and then treated the mice with aerosolized sphingosine at this time to determine therapeutic efficacy. To gain a better understanding of the mechanism underlying sphingosine-mediated bacterial killing, we determined bacterial outer and inner membrane permeabilization, inner membrane potential and intracellular acidification upon *in vitro* sphingosine treatment.

Materials and Methods

Mouse housing

CF-1 outbred mice were obtained from Charles River Laboratories (Wilmington, MA). Male mice aged 6-8 weeks were used for experiments and randomized into experimental groups. Mice were housed in standard environmental conditions with corn-cob bedding in groups of four. A standard pellet diet and water was provided *ab libitum*. Mice were acclimated for at least one week prior to experimentation. All murine experiments were performed between 8 AM and 1 PM.

Cecal ligation and puncture

A non-lethal CLP (mortality rate less than 10 %) was used to induce polymicrobial sepsis as previously described [21, 22]: Briefly, mice were anesthetized with 2% isoflurane in oxygen, shaved over the abdominal area, disinfected with povidone-iodine, and placed supine on a heating pad. Following a 1 to 2 cm midline laparotomy incision, 33 % of the cecum was ligated with a 4-0 silk tie (Syneture, Norwalk, CT). The cecum was punctured on the anti-mesenteric side with a 25-gauge needle and a small amount of feces was extruded through the puncture hole to ensure perforation. The cecum was replaced in its original location, and the midline incision was closed in two layers with 4-0 silk. Following CLP, mice were resuscitated with 1 mL sterile normal saline (NS), (Hospira, Lake Forest, IL, USA) injected subdermally and monitored to ensure that they wake up from the anesthesia. They were subsequently allowed to recover on a 42.0°C heating pad for one hour. Mice were then returned to their home cage and monitored for any complications twice a day until the experiment was completed. Sham mice underwent the above process except for ligation and puncture.

Pseudomonas aeruginosa infection

We used *P. aeruginosa* strain 762, which was isolated from a septic patient (strain fist described in [23]). The strain is non-mucoid and multidrug-resistant. Bacteria from frozen stocks were plated on fresh tryptic soy agar (TSA) plates (BD Biosciences, Franklin Lakes, NJ) and cultured overnight at 37°C. Bacteria were then resuspended in 40 mL Trypticase soy broth medium. The inoculated medium was incubated for 1 h at 37°C with shaking at 125 rpm. Bacteria were washed twice and resuspended in PBS to a concentration of 50×10^6 Colony Forming Units (CFU)/mL based on a standard absorption curve. Mice were then infected with 10^6 CFU *P. aeruginosa* 762 (20 μl) intranasally under short anesthesia with 3 % isoflurane using a 31-gauge blunted needle. Mice were returned to their home cage and monitored for any complications twice a day until the experiment was completed. Non-infected mice underwent the same process, but were administered PBS.

Sphingosine immunofluorescence analysis

Sphingosine staining was performed as previously described [13, 19]. Mice were euthanized with CO_2 at a 30 % fill rate 4 h after infection and lungs were inflated with 1 mL 10 % neutral buffered formalin (Thermo Fisher Scientific, Waltham, MA), harvested and fixed in 10 % neutral buffered formalin for 48 h. The tissue was then dehydrated with ethanol, embedded in paraffin and sectioned at 5-10 μm. Sections were dewaxed and rehydrated. Antigen-retrieval was conducted with Pepsin Digest All (Invitrogen, Thermo Fisher Scientific) at 37°C for 30 min followed by blocking with 5 % Fetal Calf Serum (FCS). Sections were stained with monoclonal mouse anti-sphingosine antibody (clone NHSPH, Alfresa Pharma Corporation, Osaka, Japan) followed by Cy3-coupled donkey anti-mouse IgM F(ab)$_2$ fragments (polyclonal, RRID AB_2340815, Jackson ImmunoResearch, West Grove, PA). Sections were washed, embedded with Mowiol and analyzed on a Leica confocal microscope (Leica, Wetzlar, Germany). 3-4 sections per group were analyzed and 15-20 vision fields of tracheal epithelial cells per section were imaged at 400x magnification. The sphingosine signal in these images was quantified using ImageJ software (NIH, Bethesda, MD). The specificity of the anti-sphingosine antibody has been previously described. Briefly, binding to ceramide, sphingosine-1-phosphate, sphinganine, sphingomyelin, cerebroside, gangliosides, phosphatidylserine, lyso-phosphatidylserine and lyso-phosphatidylcholine has been excluded [13].

Determination of bacterial load

Mice were euthanized with CO_2 at a 30 % fill rate 4 h after infection with *P. aeruginosa* 762 and bronchoalveolar lavage (BAL) fluid was harvested: 1 mL of sterile normal saline was flushed through the trachea into the lungs with a 20-gauge catheter as previously described [19]. Approximately 0.7 mL of fluid was then recovered through the same syringe. Samples were serially diluted in sterile normal saline and cultured on TSA plates at 37°C. Colonies were counted the next morning.

In vitro antimicrobial activity

Bacteria were prepared as described above for both *P. aeruginosa* 762 and ATCC19660 strains. Sphingosine was diluted in normal saline and sonicated 10 min prior to use. 10^6 CFUs were treated with 100 μM sphingosine or normal saline for 4 h at 37°C. Samples were then diluted in normal saline, plated on TSA plates and incubated at 37°C. Colonies were counted the next day.

Core body temperature monitoring

An Anipill temperature-monitoring implant (Data Science International, New Brighton, MN) was inserted into the abdomen during the CLP surgery. Core temperature was recorded electronically every 15 min until the mice were sacrificed.
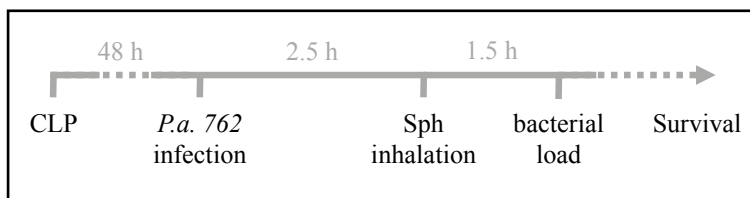
Sphingosine inhalation

Mice were stratified into two groups based on their weight loss after CLP surgery. One group received aerosolized normal saline and the other aerosolized sphingosine. In preparation, sphingosine (D-erythro-sphingosine [d18:1], Tocris, Minneapolis, MN) was diluted to 125 μM in normal saline and sonicated for 10 min. Mice were restrained in a Buxco Inhalation Tower (Data Science International) and inhaled 333 μl/mouse 125 μM sphingosine for 15 min at -0.25 cm H_2O pressure and with an In Flow of 0.25 l/min/mouse 2.5 h after infection with *P. aeruginosa* 762. The time for inhalation was chosen based on the results of core body temperature monitoring in response to pulmonary infection with *P. aeruginosa* 762. Fig. 1 summarizes the experimental timeline.

Assessment of outer membrane permeabilization

Outer membrane permeabilization was assessed using *N*-phenyl-1-naphtylamine (NPN) as previously described [24]. Briefly, when the integrity of the outer membrane of Gram-negative bacteria is disturbed, its barrier properties are compromised and NPN accumulates in its hydrophobic core, resulting in increased fluorescence [25-27]. Bacteria (*P. aeruginosa* 762) were prepared as described for pulmonary infection. 3×10^6 CFUs were plated in 96 well plates. Sphingosine was sonicated for 10 min. NPN was added to a final concentration of 10 μM. Sphingosine was added to a final concentration of 100 μM sphingosine. Positive controls were treated with ethanol at a final concentration of 67 % to determine maximum NPN uptake and blanks without bacteria were assessed to subtract background fluorescence. Measurements were made using a Cytation 5 microplate reader (BioTek, Winooski, VT) and initiated immediately after compound addition. The signal was read every minute for five minutes for four wells per group and results were averaged. Background fluorescence was subtracted and the signal obtained for ethanol-treated samples defined as 100 %. NPN uptake was then calculated for sphingosine and vehicle-treated samples.

Fig. 1. Timeline of determining the therapeutic potential of sphingosine treatment of established pneumonia. Mice underwent CLP surgery and a temperature monitoring probe inserted into the abdomen at the time of CLP. 48 h later, mice were infected intranasally with *P. aeruginosa* 762 and inhaled with sphingosine 2.5 h after infection. Bacterial loads in the lung were determined 1.5 h after sphingosine inhalation, corresponding to 4 h after infection.

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Assessment of inner membrane permeabilization

Inner membrane permeabilization was assessed using propidium iodide (PI). PI fluorescence increases upon binding to nucleic acids, but PI cannot cross intact membranes. Bacteria (*P. aeruginosa* 762) were prepared as described for pulmonary infection. 2×10^5 CFUs were plated in 96 well plates. Sphingosine was sonicated for 10 min. PI (ThermoFisher Scientific) was added to a final concentration of 60 µM. Sphingosine was added to a final concentration of 100 µM sphingosine. Positive controls were treated with ethanol at a final concentration of 67 % to determine maximum PI uptake. Measurements were made using a Cytation 5 microplate reader (BioTek) every 15 minutes over 4 h. The signal obtained for ethanol-treated samples was defined as 100 %. PI uptake was then calculated for sphingosine and vehicle-treated samples.

Assessment of membrane potential

Gram-negative bacteria normally maintain an electrical potential gradient across the inner membrane, which is crucial for a number of physiological processes, including ATP synthesis [28]. $DiOC_2(3)$ and JC-1 are positively charged lipophilic probes with green fluorescence that bind to the inner side of the inner membrane in actively growing cells [28]. At higher concentrations, aggregates form in the cytoplasm that result in a shift towards red fluorescence [28]. The ratio of red to green fluorescence can be used to determine bacterial membrane potential and a flow cytometry-based assay has been previously described [29]. Briefly, *P. aeruginosa* 762 were prepared as described above and stained with 30 µM $DiOC_2(3)$ (ThermoFisher Scientific) or 2 µM JC-1. Sphingosine was sonicated for 10 min and then added to the samples for 30 min at a concentration of 100 µM. JC-1 was added to the samples for the final 10 min of incubation with sphingosine. Controls were treated with normal saline. Fluorescence was measured on an Attune NxT flow cytometer (ThermoFisher Scientific) upon excitation with a blue 488 nm laser and detected through 530/30 nm (green) and 590/40 nm (red) emission filters.

Assessment of intracellular acidification

DND-189, also known as LysoSensor Green, is an acidotropic, membrane-permeable dye that can be used to track pH changes in living cells, as its fluorescence increases in acidic environments [30]. Bacteria (*P. aeruginosa* 762) were prepared as described above and stained with 1 µM DND-189 (ThermoFisher Scientific). Sphingosine was sonicated for 10 min and then added to the samples for 30 min at a concentration of 100 µM. Controls were treated with normal saline. Fluorescence was measured on an Attune NxT flow cytometer (ThermoFisher Scientific) upon excitation with a blue 488 nm laser and detected through 530/30 nm (green) emission filter.

Statistical analysis

Prism software (GraphPad, La Jolla, CA) was used for all statistical comparisons. Survival differences were assessed using log-rank test. ROUT method was used to test for statistically significant outliers and data points were removed when the criteria were met (false discovery rate Q = 1 %). For comparisons between two groups, Student t-test was used, and for multiple comparisons 1-way analysis of variance (ANOVA) with the indicated *post-test*. Data are graphed as mean + standard error of the mean. A p-value of $p \leq 0.05$ was considered statistically significant.
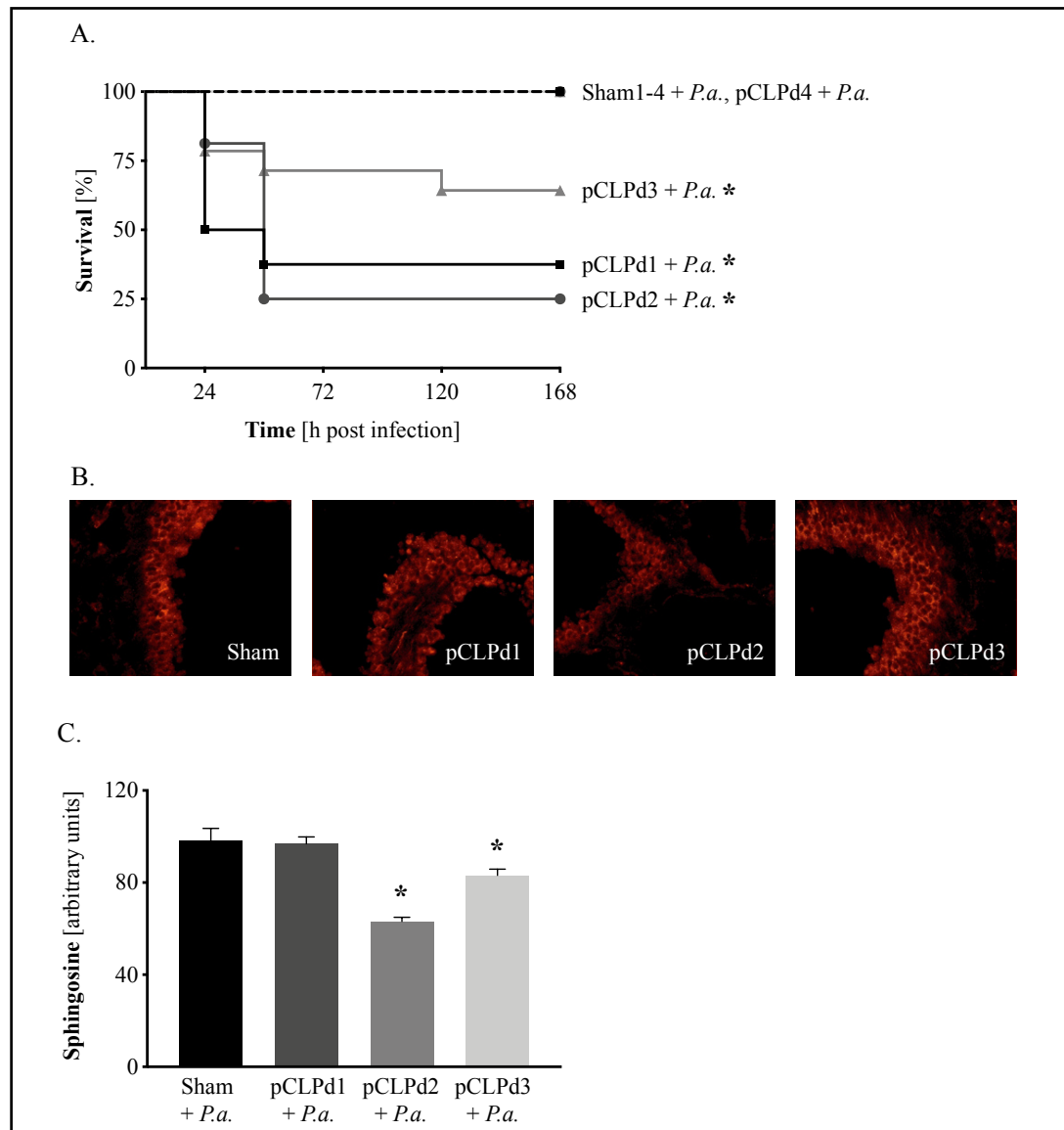
Results

Septic mice are susceptible to pulmonary P. aeruginosa infection up to three days after CLP and their airway sphingosine levels are reduced

Pulmonary infections with *P. aeruginosa* are a major cause of morbidity and mortality in hospitalized patients [31]. Septic patients, in particular, are vulnerable to *P. aeruginosa* pneumonia [32]. Antibiotic resistance is a leading cause for antibiotic therapy failure, so novel therapeutics are direly needed. Sphingosine inhalation has been shown to protect mice from pneumonia [13, 16-19]. However, it is currently still unclear if it can mitigate an established pulmonary infection. We first tested when after sepsis mice are susceptible to lung infection. For this, we infected mice 1 day, 2 days, 3 days and 4 days after CLP surgery. Sham operated mice were infected on respective days and assessed as controls. We did not observe mortal-

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ity in any of the sham-operated cohorts or in the septic mice that were infected on day 4 (Fig. 2A). For the other septic cohorts, the mortality rates were approximately 60 % for infection on day 1, 75 % for infection on day 2 and 40 % for infection on day 3 (Fig. 2A).

The susceptibility to pneumonia has previously been linked to reduced levels of sphingosine in the airways [13, 16-19]. To test for a possible reduction of sphingosine levels in our septic mice, we harvested lungs on those days after surgery when mice had shown susceptibility to *P. aeruginosa* infection (pCLPd1-3) and quantified sphingosine in the respiratory

Fig. 2. Septic mice are susceptible to pulmonary *P. aeruginosa* infection up to three days after CLP and their airway sphingosine levels are reduced. (A) Mice were subjected to cecal ligation and puncture (CLP) and inoculated intranasally with 10^6 CFU *P. aeruginosa* 762 one, two, three or four days later. Survival was monitored for 7 days after infection. n = 16 mice/group (days 1 and 2), n = 14 mice/group (day 3), n = 8 mice/group (day 4) spread across two biologically independent experiments. * p<0.05 vs respective Sham + *P.a.* (log-rank test). (B+C) Mice were subjected to cecal ligation and puncture (CLP). One, two or three days after sham or CLP surgery, in the absence of pulmonary infection, sphingosine levels in tracheal epithelial cells were analyzed by immunofluorescence (400x magnification). (B) A representative section is shown for each group. (C) The sphingosine signal in the respiratory epithelium was quantified using ImageJ. n = 3-4 mice/group and 15-20 vision fields/mouse were analyzed. * p<0.05 vs Sham (ANOVA with Dunnett posttest).

epithelium by immunofluorescence staining (Fig. 2B). Septic mice had significantly reduced sphingosine levels in their respiratory epithelium on day 2 and day 3 after CLP surgery, with pCLPd2 showing the most pronounced reduction by approximately 35 % (Fig. 2C).

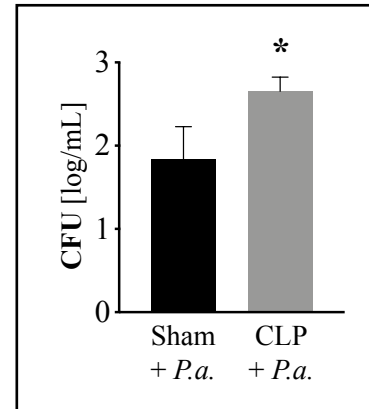### Septic mice have higher pulmonary bacterial loads upon P. aeruginosa infection two days after CLP

Given that the highest mortality rate was observed in the post-CLP day 2 (pCLPd2) cohort, we determined lung bacterial loads in these mice by harvesting BAL fluid 4 h after infection. The bacterial burden in septic mice was approximately 10-fold higher than in sham-operated controls (Fig. 3). In light of the high mortality rate and significant bacterial load in pCLPd2 mice upon infection, we chose this time point for subsequent experiments.

Sphingosine has antimicrobial activity against clinical P. aeruginosa isolates

Sphingosine has been reported to exhibit antimicrobial properties against a wide range of Gram-positive and Gram-negative bacteria. Reports on its effectiveness against *Pseudomonas* strains have varied [11, 13]. To confirm that sphingosine can kill the multi-drug resistant *Pseudomonas* isolate used in our study, we treated bacteria with 100 µM sphingosine *in vitro* for 4 h. This time-point was chosen as it corresponds to the time at which we determined bacterial loads in the airways after infection of septic mice (Fig. 2B). The samples were then diluted and plated to determine CFUs. Even when significantly lower dilutions for the sphingosine samples were plated (1:10 rather than 1:100,000) no colonies were observed for *P. aeruginosa* 762, indicating more than 99.999 % of bacteria were killed (Fig. 4A). The antimicrobial activity against another strain, ATCC19660, was not quite as pronounced, averaging 72 % (Fig. 4B).
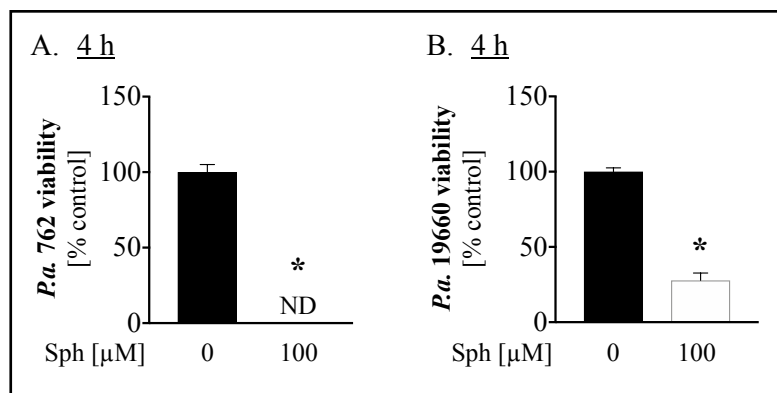
Fig. 3. Septic mice have higher pulmonary bacterial loads upon *P. aeruginosa* infection two days after CLP. Bacterial loads on the airways were determined 4 h after infection by bronchoalveolar lavage. n = 6-8 mice/group. * p<0.05 vs Sham + *P.a.* (one-tailed Student t test).

Sphingosine inhalation rescues septic mice from an established lung infection

In order to determine at what time after inoculation septic mice develop a lung infection, we continuously monitored core body temperature. The CLP surgery led to an initial drop of core body temperature, which had normalized in the two days prior to the *P. aeruginosa* inocula-

Fig. 4. Sphingosine has antimicrobial activity against clinical P. aeruginosa isolates. Sphingosine was added to *P. aeruginosa* 762 (A) or *P. aeruginosa* ATCC19660 (B) at a final concentration of 100 µM. Samples were incubated for 4 h, then diluted and plated overnight. CFUs were counted the next day. n = 9 samples/group, spread across 3 biologically independent experiments. p < 0.05 vs 0 µM (Student t test).

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Fig. 5. Sphingosine inhalation rescues septic mice from an established lung infection. (A) Mice were subjected to cecal ligation and puncture (CLP) and a temperature monitoring probe was inserted into the abdomen at time of the procedure. Core body temperature was electronically monitored every 15 minutes starting at the time of CLP. On d2 after CLP, mice were inoculated with 10^6 CFU *P. aeruginosa* 762. Monitoring of core body temperature continued until the mice were sacrificed 20 h after the infection. n = 7 mice.
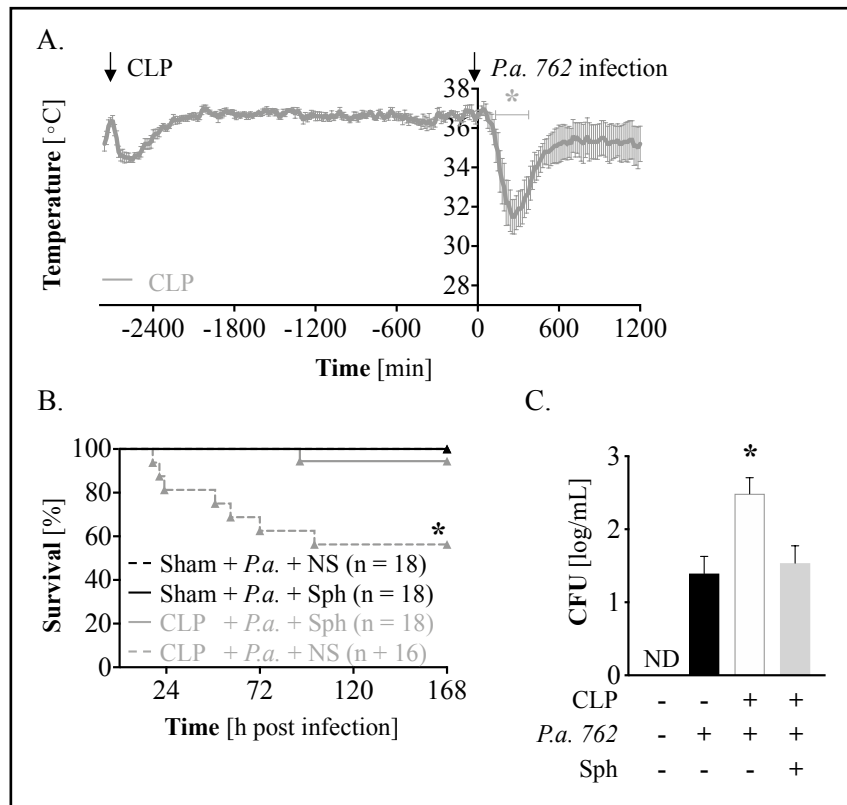
* $p<0.05$ vs temperature at time 0 (two-way repeated-measures ANOVA with Dunnett posttest). (B) Mice underwent CLP and were infected with *P. aeruginosa* 2 days later. 2.5 h after infection, mice were inhaled with either sphingosine or normal saline and survival was monitored. n = 16-18 mice/group spread across three biologically independent experiments. * $p<0.05$ CLP + P.a. + NS vs all other groups (log-rank test). (C) Airway bacterial loads were determined by bronchoalveolar lavage 90 minutes after sphingosine or normal saline inhalation. ND: not detected. n = 18-20 mice/group spread across three biologically independent experiments. * $p<0.05$ CLP(+) P.a.(+) Sph(-) vs all other groups.

tion (Fig. 5A). Core body temperature started to drop again approximately 90 min after inoculation and remained significantly reduced between 2.5 h and 7.75 h after infection (Fig. 5A).

Based on the observed temperature drop, mice were inhaled with either normal saline or sphingosine 2.5 h after inoculation and subsequently monitored for survival. Bacterial loads were again determined in BAL fluid 4 h after the inoculation (90 minutes after sphingosine inhalation, please refer to Fig. 1 for experimental timeline). As before, sham mice showed no mortality from *P. aeruginosa* infection irrespective of treatment (Fig. 5B). Septic mice that received aerosolized normal saline had a 43.8 % mortality rate, whereas only one septic mouse that was treated with aerosolized sphingosine died, corresponding to mortality rate of only 5.6 % (Fig. 5B). Regarding bacterial loads, sphingosine inhalation reduced the bacterial loads approximately 10-fold compared to inhalation with normal saline, and actually lowered them to the levels found in healthy mice infected with *P. aeruginosa* (Fig. 5C).

Sphingosine permeabilizes the outer membrane and disrupts inner membrane potential
Membrane permeabilization has been suggested to underlie the antimicrobial activity of sphingosine [14, 15]. To determine if sphingosine induces outer membrane permeabilization in *Pseudomonas*, we incubated bacteria with sphingosine in the presence of NPN. When the integrity of the outer membrane of Gram-negative bacteria is compromised, its barrier properties are disturbed and NPN can accumulate in its hydrophobic core, which increases

NPN fluorescence [25-27]. Within 5 minutes of sphingosine addition, NPN fluorescence was significantly increased to 35 % of its signal upon complete membrane disruption (Fig. 6A).

To test for the kinetics of inner membrane permeabilization, we utilized PI. Intact membranes are impermeable for PI, whose fluorescence increases upon binding to nucleic acids. Sphingosine caused a significant increase in PI fluorescence 75 minutes after its addition (Fig. 6B).

To determine if the function of the inner membrane is compromised, we assessed membrane potential. Gram-negative bacteria normally maintain an electrical potential across the inner membrane, which is crucial for a number of physiological processes, including ATP synthesis [28]. $DiOC_2(3)$ and JC-1 are positively charged lipophilic probes with green fluorescence that bind to the inner side of the inner membrane in actively growing cells [28]. At higher concentrations, aggregates form in the cytoplasm that result in a shift towards red
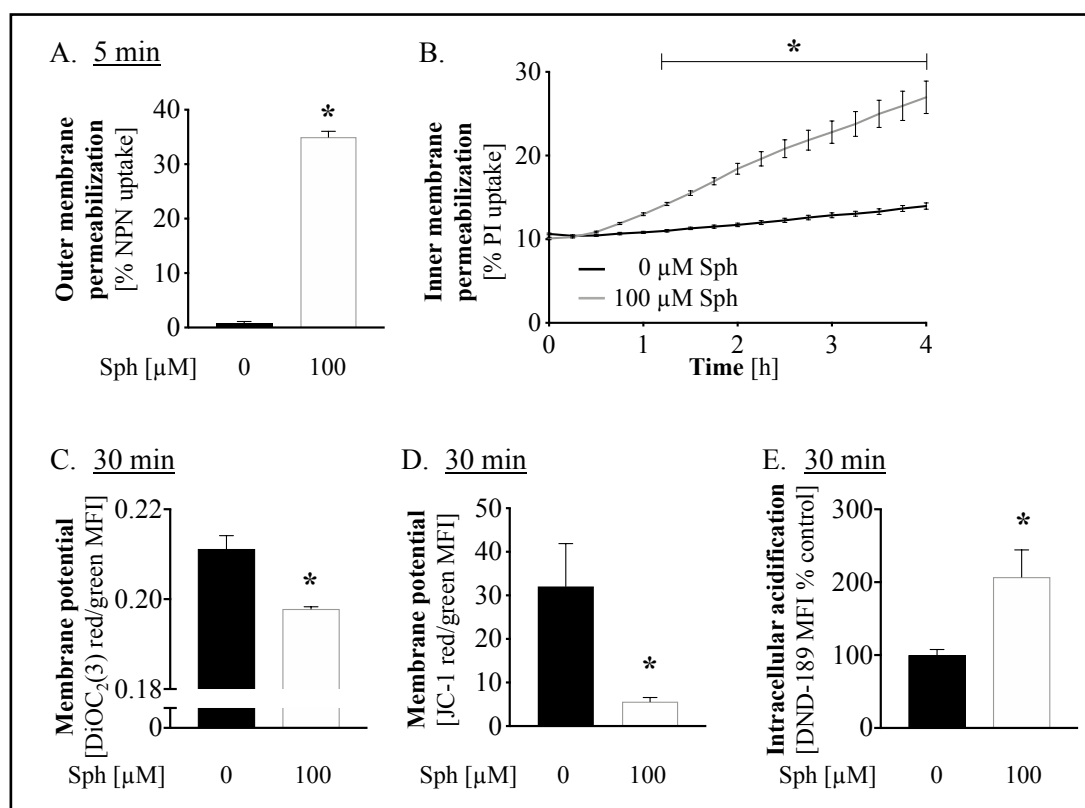
Fig. 6. Sphingosine permeabilizes the outer membrane and disrupts inner membrane potential. (A) Outer membrane permeabilization was determined by NPN uptake in *P. aeruginosa* 762. Bacteria were treated with normal saline or 100 μM sphingosine and NPN fluorescence measured using a microplate reader. 100 % NPN uptake was defined as the signal obtained upon treatment of bacteria with 67 % ethanol. n = 12 samples/group, spread across three independent experiments. * p<0.05 vs 0 μM (Student t test). (B) Inner membrane permeabilization was determined by PI uptake in *P. aeruginosa* 762. PI fluorescence was measured over 4 h after sphingosine addition using a microplate reader. 100 % PI uptake was defined as the signal obtained upon treatment of bacteria with 67 % ethanol. n = 12 samples/group, spread across three independent experiments. * p<0.05 vs 0 μM (two-way repeated-measures ANOVA with Dunnett posttest). (C+D) Membrane potential was determined by $DiOC_2(3)$. (C) and JC-1 (D) fluorescence shift from green to red using flow cytometry. Samples were analyzed 30 min after sphingosine addition. n = 6 samples/group, spread across two independent experiments. * p<0.05 vs 0 μM (Student t test). (E) Changes in intracellular pH were measured with DND-189 in *P. aeruginosa* 762. DND-189 fluorescence was measured by flow cytometry 30 min after sphingosine addition. n = 9 samples/group, spread across three independent experiments. * p < 0.05 vs 0 μM (Student t test).

fluorescence [28]. The ratio of red to green fluorescence can be used to determine bacterial membrane potential. We noted a decrease in the fluorescence ratio of both $DIOC_2(3)$ (Fig. 6C) and JC-1 (Fig. 6D) 30 minutes after sphingosine addition, indicating reduced dye uptake and thus a reduction of the membrane potential.

Disturbances in membrane potential indicate an altered permeability for ions. This, in turn, is crucial for maintaining pH homeostasis [33]. Failure to maintain an appropriate pH has deleterious effects on the activity of biological macromolecules, significantly reducing growth and viability [34]. To see if the disturbance of the membrane potential resulted in intracellular acidification, we used DND-189. This compound, more commonly known as LysoSensor Green, is a membrane-permeable dye with pH-sensitive fluorescence [30]. We noted that the fluorescence of DND-189 approximately doubled 30 minutes after sphingosine addition to the bacteria (Fig. 6E).

Discussion

Using a 2-hit model of sepsis and subsequent nosocomial infection (Fig. 1), our data demonstrate that septic mice are most susceptible to *P. aeruginosa* lung infection two days after sepsis induction (Fig. 2A). This coincided with a reduction of sphingosine in the tracheal epithelium (Fig. 2B+C). Sphingosine killed 99.999% of bacteria of the multi-drug resistant, clinical *P. aeruginosa* isolate used for the lung infection *in vitro* (Fig. 4A). To tests sphingosine's efficacy *in vivo*, we determined at what time after inoculation mice have established pneumonia. Based on a significant decrease in core body temperature, this was 2.5 h after inoculation (Fig. 5A). When inhaled sphingosine was administered at this time, the airway bacterial burden in septic mice was reduced to the levels found in healthy mice after inoculation (Fig. 5C). This was associated with a significant survival benefit (Fig. 5B).

Previous studies have established that sphingosine can be inhaled preventively, to restore reduced sphingosine levels in susceptible mice. For instance, using a protocol with a net sphingosine inhalation comparable to our method, Pewzner-Jung *et al.* reported that sphingosine inhalation of cystic fibrosis mice increases sphingosine levels in respiratory epithelial cells from approx. 3 pmol/10^5 cells in control-inhaled mice to 11 pmol/10^5 cells in sphingosine-inhaled mice, which corresponds to the level found in healthy mice [13]. This and other studies have shown that this is sufficient to protect susceptible mice from a subsequent infection [13, 16-19].

Which step in sphingolipid metabolism is affected and results in the reduction of sphingosine in susceptible mice is currently unknown and may differ based on the context (i.e. cystic fibrosis, trauma/sepsis). PAR1-SIP3 signaling has been reported to amplify inflammation in septic mice, suggesting that sphingosine phosphorylation may be enhanced. However, the study focused on dendritic cells, not the airway and sphingosine-1-phosphate levels were not quantified [35]. On the other hand, sphingosine reductions in cystic fibrosis mice have been linked to downregulation of acid ceramidase (ASAH1) [36]. We have also observed an increase in ceramide levels in the airways of critically ill mice (data not shown). This is a further commonality between critically ill and cystic fibrosis mice and indicates that the defect is with sphingosine production, rather than with sphingosine conversion to sphingosine-1-phosphate. However, further studies are necessary to elucidate the exact molecular mechanisms underlying the decrease of sphingosine levels in septic mice.

So far, it has not been investigated how long the prophylactic effect of sphingosine inhalation lasts. Additionally, it remained unknown if sphingosine would also be beneficial when administered after an infection. Our study addressed the second question. We can now show that sphingosine is also effective when given therapeutically, rather than preventively. We tested the efficacy of sphingosine in treating a pulmonary infection with a multidrug-resistant *P. aeruginosa* strain, which is of particular clinical importance, since *P. aeruginosa* is frequently the cause of hospital-acquired pneumonia and bacterial resistance is a leading cause of treatment failure. Antimicrobial lipids, such as sphingosine, have garnered much

interest in the last years, as resistance to classical antibiotics is an increasing problem in virtually all microbes (reviewed in [37]).

Sphingosine is also a promising new treatment, because it has been reported to kill many different Gram-positive and Gram-negative bacteria *in vitro*. In this current study, we did not assess whether therapeutic sphingosine is also effective against pathogens other than *P. aeruginosa*. However, the previous prophylactic *in vivo* studies have also shown effectiveness against different pathogens, including *S. aureus*, another common hospital-acquired pathogen [13, 16-19].

Preliminary results indicate that side effects from sphingosine inhalation are likely to be minimal: No significant signs of toxicity in the respiratory epithelium were found upon sphingosine inhalation in mice [20] and mini pigs [7]. Additionally, sphingosine is a natural part of the host defense of the airways and has been reported to be reduced in cohorts that are susceptible to pulmonary infection [13, 16-19]. Thus, when dosed correctly, inhalation with sphingosine or sphingosine donors restores the pathologic loss, but does not increase it above normal levels [18]. Further, there are some indications that sphingosine inhalation may even mitigate tissue injury by reducing inflammatory cytokine levels [13, 17].

Although the antimicrobial activity of sphingosine was first described in 1992 [10], little is still known regarding the underlying mechanism. Studies with artificial liposomes and ghost membranes have shown that sphingosine can permeabilize membranes [14, 15]. In line with this Fischer *et al.* observed changes in the cell surface of sphingosine-treated bacteria using electron microscopy [12]. While the tested Gram-positive bacteria (*Staphylococcus aureus*) showed signs of membrane disruption, the cytoplasmic membrane of the tested Gram-negative bacteria (*Escherichia coli*) appeared intact [12]. Taken together, this indicates that sphingosine may kill Gram-positive bacteria through membrane permeabilization, but how death of Gram-negative bacteria is induced if the inner membrane stays intact remains unclear. To gain further insight, we first assessed outer membrane permeabilization in *Pseudomonas*, which occurred within 5 min of sphingosine addition (Fig. 6A). Inner membrane permeabilization, however, was not evident until 65 min after sphingosine addition (Fig. 6B). The integrity of the outer membrane contributes to the maintenance of an electronic gradient across the inner membrane [38]. We noted a disruption of inner membrane potential 30 min after sphingosine treatment (Fig. 6C+D). Preserving the correct membrane potential is critical for many physiological processes, including ATP synthesis and ion transport. The latter, in turn, is crucial for maintaining pH homeostasis. Failure to prevent intracellular acidification severely effects the activity of biological macromolecules, significantly reducing growth and viability [34]. In line with a disturbance of membrane potential, we also noted increased intracellular acidification upon sphingosine treatment (Fig. 6E). Taken together, these data indicate that sphingosine kills *Pseudomonas* by rapidly permeabilizing the outer membrane, resulting in loss of membrane potential and increased intracellular acidification. Bacteria can have protective strategies for dealing with acidic stress, e.g. the acid tolerance response [34]. As these may vary between strains, this could explain the different efficacies of sphingosine-mediated bacterial killing observed in our study. Further, outer membrane composition may influence the susceptibility to permeabilization by sphingosine [15, 39]. Further studies will be necessary to elucidate the mechanism underlying sphingosine's antimicrobial properties.

It should be noted that relatively high concentrations of sphingosine were used in the present studies, while previous studies showed bacterial death already at sphingosine concentrations between 1 and 10 μM (4) and a more rapid onset of bacterial death [40]. However, our study also used considerably higher bacterial cell counts. Thus, the principal effects of sphingosine are similar in all of these studies, but the concentrations required for induction of death and the antimicrobial kinetics seem to slightly differ between strains, growth and medium conditions.

The administered sphingosine is not inert and thus could be metabolized further. We cannot exclude that the observed beneficial effects of sphingosine inhalation are mediated by a metabolite rather than by sphingosine itself. However, sphingosine inhalation does not

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seem to increase ceramide and sphingosine-1-phosphate levels [7]. Further, sphingosine inhalation was shown to increase sphingosine levels in the respiratory surface, but not the epithelial cell layer [7] and it has been suggested that sphingosine mostly remains in the mucus on top of the epithelial cells [40]. This would favor direct bacterial killing by sphingosine.

The use of already clinically approved drugs could potentially accelerate the translation of these findings to clinical use. Fingolimod, an approved sphingosine-1-phosphate receptor modulator, could be one potential option. However, as long as the molecular mechanisms have not been elucidated, we can only speculate on the effect of Fingolimod. The observation that ceramide is increased in critically ill mice indicates that the defect is with sphingosine production, rather than consumption. Further, direct antimicrobial properties of Fingolimod have so far only been demonstrated for *Clostridium perfringens* [41]. Assuming a direct bactericidal effect of sphingosine, as discussed above, the reconstitution of these bacterial killing properties is likely paramount to mitigate pulmonary infection in the model. Rather than Fingolimod, the use of sphingomyelinase inhibitors like Amitriptyline, are another promising option when it comes to already approved drugs. These substances are of particular interest for those situations in which sphingosine reductions occur concurrently with ceramide elevations, as ceramide elevations were shown to be responsible for the reduced expression of acid ceramidase and the subsequent reduction of sphingosine [36]. Indeed, two phase II studies with pulmonal amitriptyline already reported with favorable results [42]. Nevertheless, clinical development of sphingosine inhalation itself is also underway at least [7] and could potentially garner a lot more attention soon, as sphingosine was very recently shown to prevent viral entry of SARS-CoV-2 [43].

Conclusion

Our data provide proof-of-principle that sphingosine inhalation can be used to treat an established pulmonary infection with *P. aeruginosa*. Further studies are needed to test whether sphingosine is also effective as a therapeutic against infections with other bacteria.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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