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Bronchoalveolar Lavage Microvesicles Protect Burn-Injured Mice from Pulmonary Infection

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Abstract

Background—*Pseudomonas aeruginosa* (*PA*) is a major cause of morbidity and mortality among burn patients, despite antibiotic therapy. There is a need to identify innate immune defenses that prevent *PA* infection in injured adults in an effort to find therapeutic alternatives to antibiotics. Here, we tested our hypothesis that Microvesicles (MVs) in bronchoalveolar (BAL) fluid have a role in the immunity of the lung in response to pathogens.

Study Design—MVs were isolated from murine BAL fluid, quantified using nanoparticle tracking analysis, and injected into burn injured mice prior to *PA* infection. Survival was assessed and BAL bacterial loads enumerated. Neutrophil number and IL-6 activity were determined. Lungs were harvested and sphingosine (SPH) content analyzed via immunohistochemistry. Antimicrobial effects of MV and SPH enriched MVs were assessed in an *in vitro* assay.

Results—Burn injured mice have reduced BAL MV number and SPH content as compared to sham. When BAL MVs from healthy mice are administered to injured mice, survival and bacterial clearance are robustly improved. We further observed that intranasal administration of MVs restores SPH levels after burn injury, MVs directly kill bacteria, and this bacterial killing is increased when the MVs supplemented with SPH.

Conclusion—Using a pre-clinical model, BAL MVs are reduced after scald injury and BAL MV restoration to injured mice improves survival and bacterial clearance. The antimicrobial mechanisms leading to improved survival includes the quantity and SPH content of BAL MVs.

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Introduction

Pneumonia is the leading cause of mortality resulting from nosocomial infections in patients admitted to intensive care units¹. Mechanically ventilated patients are particularly vulnerable to pneumonia infection. Ventilator-associated pneumonia is thought to be caused by the endotracheal tube bypassing the innate immune defenses of the upper respiratory tract allowing bacteria into the lung². *Pseudomonas aeruginosa* (*PA*) is a virulent gram negative pathogen that has the ability to form a biofilm on the inside of endotracheal tubes increasing the likelihood of developing pneumonia³. It is more prevalent in susceptible patient populations, including immunosuppressed, cystic fibrosis, and burn patients⁴. With improvements in wound care and resuscitation, pneumonia is a major cause of morbidity and mortality in the burn patient population^{1,5,6}. In particular, *PA* is the most frequently isolated bacterial pathogen^{1,7}. Further complicating *PA* treatment is rising antibiotic resistance, contributing to higher mortality rates^{3,4,6}. Due to the high associated mortality, there is a need to identify innate immune defenses that prevent *PA* infection in healthy adults, in an effort to find a therapeutic alternative or complement to antibiotics.

Sphingosine (SPH) is a lipid found in a variety of cell membranes and cell membrane-derived microvesicles (MVs) that has antimicrobial properties⁸. It has been shown to contribute to innate antimicrobial activity of skin and mucosal surfaces^{9–11}. In particular, it has been shown to protect skin from bacterial colonization¹², lungs from pulmonary infection in cystic fibrosis⁸ and burn injury models⁶. SPH is generated as the product of hydrolysis of sphingomyelin to ceramide via sphingomyelinase and subsequently ceramide to SPH via the enzyme ceramidase^{8,11}. The susceptibility to pulmonary infection seems to be related to the lipid composition of the cell membranes, with alterations in the ceramide/sphingosine pathway resulting in altered immunity^{6,8}. Ceramide levels have been shown to be pathologically increased whereas SPH levels are decreased in tracheal epithelial cells of cystic fibrosis and burn-injured murine models^{6,8}. Prophylactic treatment with aerosolized SPH decreased bacterial load and improved survival in these studies^{6,8}. However, the role of tracheal epithelial cell derived MVs, which inherently contain SPH, in the prophylactic treatment of *PA* pulmonary infection has not been investigated.

MVs are small vesicles derived from cell membranes of heterogeneous density and composition¹³. During cellular activation and apoptosis MVs are known to transfer information via cell-to-cell communication^{14,15}. While found in healthy individuals, MV quantity and type are altered during inflammatory disease^{16–18}. MV signals can be mediated by a variety of molecules including protein, lipids, and carbohydrates. These signals depend largely on the parent cell, which can be lymphocytes, platelets, leukocytes, endothelial and epithelial cells^{14,15}. Platelet-derived MVs are the most abundant whereas neutrophil-derived MVs are the dominant type generated at the site of infection^{14,19,20}. Neutrophil-derived MVs have been shown to increase the inflammatory response and increase mortality^{14,19}. While it is known that MVs in bronchoalveolar (BAL) fluid have a role in the immunity of the lung in response to pathogens, the mechanism is unknown¹⁵.

The role of BAL MVs in response to *PA* pneumonia in burn patients has not been investigated. Given previous data in the burn-injured murine model, we hypothesize that

following burn injury, the quantity of BAL MVs will be reduced after burn injury and treatment of injured mice with BAL MVs from healthy donors will improve survival and bacterial clearance. Further, we hypothesize that the mechanism leading to improved survival is related to the quantity and SPH content of BAL MVs.

Methods and Materials

Mice

Six-week-old male CF-1 mice were purchased from Charles River Laboratories (Wilmington, MA, USA). They were housed in standard environmental conditions allowing them to acclimate one week prior to experiments. All experiments were conducted by protocols (# 08-09-19-01) approved by the Institution Animal Care and Use Committee of the University of Cincinnati.

Burn injury

Mice were subjected to a full-thickness scald injury as previously described²¹. Briefly, mice were weighed and anesthetized with 4.5% isoflurane in oxygen prior to clipping hair from their dorsal surface. They were placed in a template according to their weight that exposed 28% total surface body area. They were immersed in 90.0°C water for 9 seconds to produce a fullthickness scald injury to the exposed surface. Following scald injury, mice received 1.5 ml of sterile normal saline via intraperitoneal injection for resuscitation and allowed to recover on 42°C heating pad for 3 hours. Sham mice received the same treatment, except they were immersed in room temperature water.

Microvesicle isolation

Microvesicles were isolated as previously described¹⁹. In brief, bronchoalveolar (BAL) fluid was collected from sham and post burn day 1 (PBD1) mice and centrifuged at $450 \times g$ for 10 minutes at room temperature to form a cell pellet. Supernatant was collected and centrifuged at $10,000 \times g$ for 10 minutes at room temperature to remove any residual cells. Supernatant was then centrifuged at $25,000 \times g$ for 30 minutes at room temperature to pellet MVs. MV pellets were re-suspended in 1 ml of Roswell Park Memorial Institute media (RPMI) and 1:100 MV dilutions were quantified using Nanoparticle Tracking Analysis (Nanosight, Malvern Instruments Ltd, Worcestershire, UK)²².

Bacterial preparation

Pseudomonas aeruginosa 762 strain was grown for 14 hours on a tryptic soy agar plate (BD Biosciences, Franklin Lakes, NJ, USA) in the 37°C incubator as previously described⁶. Bacteria was transferred to an Erlenmeyer flask containing 40 ml of tryptic soy broth (BD Biosciences) and incubated for 60 minutes at 37°C, 125 revolutions per minute (rpm). Bacteria was centrifuged at 2800 rpm for 10 min at 21°C, washed with 40 ml of PBS (Thermo Fischer Scientific; Waltham, MA, USA), centrifuged at same settings, and then resuspended in PBS. Bacteria was diluted to an optical density of 0.04 using the photospectrometer based on a standard absorption curve for a desired concentration of 1×10^6 bacteria in 20 μ L.

In-vivo microvesicle survival study and determining bacterial load

The BAL MVs were isolated from healthy donor mice and enumerated. One day after burn injury, mice were anesthetized with 3% isoflurane in oxygen and intranasally inoculated with 1×10^9 BAL MVs /10 μ L with a 31-gauge 1 ml syringe. Thirty minutes after MV treatment, mice were intranasally inoculated with 20 μ L of bacteria at a concentration of 10^6 *Pseudomonas aeruginosa*. A group of sham and burn-injured mice without MV treatment were also inoculated with bacteria at the same time. Survival of the three groups were observed.

Bacterial load determination

Mice were euthanized and BAL fluid was harvested 4 h after bacterial inoculation. Normal saline was instilled via the trachea into the lungs with a 20-gauge catheter. Two milliliters of PBS was instilled per mouse. Between 1.8 and 2 mL of fluid was recovered. Samples were serially diluted in sterile saline and cultured on tryptic soy agar plates. Plates were incubated overnight at 37°C, and colonies enumerated.

Flow cytometric determination of surface proteins and cytokines

As described above, BAL fluid was isolated from sham, scald-injured, and MV treated scald-injured mice and centrifuged at $450 \times g$ for 10 minutes at room temperature. Cell free BAL fluid was used to determine IL-6 (BD Biosciences) levels by cytometric bead array, performed according to the manufacturer's protocol. Cells were quantified using a Coulter AcT 10 cell counter (Beckman Coulter, Brea, CA) and then suspended in FACS buffer (PBS with 1% bovine albumin and 0.1% azide) and Fc Block (BD Pharmingen, San Jose, CA). Neutrophils were characterized by staining with Ly-6G (clone 1A8, BD Biosciences) and CD11b (clone M1/70, BD biosciences) antibodies. Cells were analyzed on Attune Acoustic Focusing Cytometer using Attune Cytometric Software v2 (Thermo Fisher Scientific).

Cecal ligation and puncture

Cecal ligation and puncture (CLP) was performed as previously described¹⁹. Briefly, mice were anesthetized with 3.0 % isoflurane in oxygen, clipped and disinfected with betadine solution. A 1 cm laparotomy incision was performed. The cecum was ligated with a 3-0 silk suture and punctured on the anti-mesenteric side with a 22-gauge needle ensuring a full thickness perforation. Prior to closure, 1×10^9 MVs isolated from BAL fluid of healthy donor mice was injected into the peritoneal cavity. The midline incision was then closed with 4-0 silk suture in two layers. Mice were resuscitated with 1 mL of sterile saline via intraperitoneal injection and allowed to recover on a heating pad for 60 minutes. Eighteen hours after CLP, peritoneal fluid and blood samples were collected from CLP and CLP-MV treated mice. Peritoneal fluid was collected by injecting 10 ml of sterile saline into the peritoneal cavity and then aspirated. Blood was collected via cardiac puncture and heparinized to prevent clotting. Samples were incubated overnight on tryptic soy agar plates and CFUs were enumerated as described above.

In-vitro microvesicle bacterial killing assays

Microvesicles were isolated from two sources, tracheal epithelial cells and BAL fluid, per protocol previously described and enumerated by Nanoparticle Tracking Analysis. 1×10^7 MVs were added to 1 ml of 2×10^3 bacteria in a 24 well plate, incubated for 60 minutes at 37°C. Subsequently, 100µL of solution was plated on tryptic soy agar plates, incubated overnight at 37°C, and CFUs enumerated as previously described⁶. For SPH enriched MVs, BAL MVs were isolated as described above and were incubated with 125 µM SPH for 1 hour, rocking, at 37°C. Following incubation, MVs were pelleted via differential centrifugation. They were again enumerated via the Nanoparticle Tracking Analysis. Finally, 1×10^9 MVs were added to bacteria plates as described above for *in vitro* killing assays.

PKH fluorescence quantification

Donor mice BAL MVs were isolated as described above and subsequently labeled with PKH-26 (Sigma-Aldrich, St. Louis, MO). Mice were anesthetized with 3% isoflurane in oxygen and a concentration of 1×10^9 PKH-labeled MVs were intranasally injected into recipient mice. Mice were euthanized and lungs were harvested, fixed in 10% neutral buffered formalin (Thermo Fisher Scientific). ImageJ software²³ was used to quantify the fluorescence via histology comparison of 10 areas per sample in the apical one third of the tracheal epithelial cells.

SPH immunohistochemical analysis

Donor mice BAL MVs were isolated as described above and 1×10^9 MVs were intranasally injected into PBD1 mice. Lungs from sham, PBD1, and PBD1 MV treated mice were harvested, fixed in 10% neutral buffered formalin (Thermo Fisher Scientific), and stained with Cy3-coupled anti-SPH antibodies (clone NHSPH; Alfresa Pharma Corporation, Osaka, Japan)⁸. ImageJ software was used to analyze the immunofluorescence via histology comparison of 10 sections per sample in the apical one-third of tracheal epithelial cells.

Statistical Analysis

Using Prism 6.0 (GraphPad Software, La Jolla, CA, USA), statistical comparisons were performed with the Mantel-Cox Log-Rank test (survival), Student t-test (2 groups), or 1-way ANOVA with the Tukey post hoc comparison (>2 groups). Data were reported as means +/- SEM for experiments containing multiple data points. A *p*-value of 0.05 was considered statistically significant.

Results

Antimicrobial effects of BAL MVs decrease bacterial load and improve survival

Burn-injury or sepsis, as an initiating factor, produces a cascade effect that induces an inflammatory response, and although direct mechanisms are not well defined, MVs are known to be mediators of this immune response^{14,15,24}. Previous data has demonstrated that PBD1 mice are more susceptible to pulmonary infection and have increased mortality as compared to healthy mice⁶. We hypothesized that the etiology of this increased susceptibility is a decrease in BAL MVs. BAL MVs were isolated from PBD1 mice and

quantified using NTA (Figure 1a). Sham mice were found to have 2.3×10^{10} MVs/ml of BAL fluid, while PBD1 mice had a 60% decrease in the total number of BAL MVs ($p < 0.05$). We then measured the SPH content of those BAL MVs and saw that there was a 40% reduction in SPH content within MVs following scald injury (Figure 1b).

Given the reduction in BAL MVs 24 hours after burn injury, we sought to determine whether administering BAL MVs to PBD1 mice would abrogate *PA* pulmonary infection. BAL MVs were isolated from healthy donor mice and 10^9 MVs were delivered intranasally to PBD1 mice. After treatment, all mice were inoculated with 10^6 *PA* and survival was monitored for seven days (Figure 2a). In the first 24 hours, *PA* infected PBD1 mice had 90% mortality. This increased to 95% mortality by day 7, as compared to 5% mortality among sham mice inoculated with *PA*. Figure 2a demonstrates that treatment with MVs significantly decreased mortality among PBD1 mice inoculated with *PA* such that mortality was 39% at 24 hours and 56% at day 7. In order to associate the increase in survival to the pulmonary bacterial load, 4 hours after inoculation with *PA* BAL fluid was collected and CFUs enumerated (Figure 2b). PBD1 mice had a 3-log increase in bacterial load compared to sham mice. Administration of MVs to PBD1 mice significantly reduced bacterial loads from untreated PBD1 mice ($p < 0.05$).

To further characterize the effect of BAL MV treatment on the inflammatory response, a cytometric bead array was used to quantify IL-6 (Figure 3a) and flow cytometry to quantify neutrophils (Figure 3b). IL-6 is a known inflammatory cytokine that has an effect on a variety of cells in the immune system, including macrophages²⁵. A previous study has shown that along with other inflammatory markers, IL-6 is elevated in patients with bacterial pneumonia²⁶. In the murine burn injury – pulmonary infection model, BAL fluid was collected 4 hours after inoculation with *PA* in sham, untreated PBD1, and MV treated PBD1 mice. IL-6 in the BAL fluid of PBD1 mice increased by 75% compared to sham mice. When PBD1 mice were treated with MVs, IL-6 was significantly reduced from PBD1 levels and equivalent to sham (Figure 3a). Further characterization of the BAL fluid to quantify the neutrophils demonstrated that although PBD1 mice had a 50% increase in neutrophils compared to sham mice, there was not a significant difference after MV treatment (Figure 3b). We concluded that BAL MVs, while decreasing inflammatory cytokines, were not a neutrophilic chemoattractant.

These data demonstrate that BAL MVs have an antimicrobial effect in the murine burn model that is protective against *PA* pulmonary infection. To further emphasize the antimicrobial characteristics of BAL MVs, we administered treatment to an intra-abdominal model of sepsis, using CLP. At the time the CLP was performed, 10^9 BAL MVs were injected into the peritoneum. Peritoneal lavage fluid (Figure 4a) and blood (Figure 4b) was collected 18 hours later and CFUs enumerated. BAL MV treatment resulted in a 2-log decrease in bacterial load locally in the peritoneum (Figure 4a) and systemically in the blood (Figure 4b) as compared to untreated CLP mice. This demonstrates the antimicrobial effects of BAL MPs are not limited to the respiratory tract, but rather are applicable to multiple injury models.

Mechanisms of antimicrobial effect of BAL MVs

These data demonstrate that BAL MVs have an important role in bacterial clearance in the upper respiratory tract. However, the mechanism of this antimicrobial effect was not well known. Here, we investigated the influence of MVs on the immune system. First, *PA* was treated with 10^7 MVs isolated from BAL fluid (Figure 5a) and tracheal epithelial cells (Figure 5b) of healthy mice to conduct an *in vitro* bacterial killing assay. BAL MVs decreased bacterial growth by 25% and tracheal epithelial cell MVs decreased bacterial growth by 20%. In contrast neutrophil-derived MVs (NDMV) enhanced bacterial growth by 60% (Figure 5c). This suggests that BAL MVs are largely derived from tracheal epithelial cells and have direct antimicrobial properties in the pulmonary system. This is in contrast to NDMV which have the opposite effect and promote bacterial growth.

Tracheal epithelial cells in the burn injury – pulmonary infection model were characterized in previous studies. Following burn injury, there was a decrease in the lipid SPH and pathologic increase in ceramide, but treatment with aerosolized SPH resulted in improved bacterial clearance and survival⁶. It is also known that MVs contain the lipid SPH⁸. Here, we hypothesized that BAL MVs are a SPH donor to bronchial epithelial cells. PKH-26 is a fluorescent, lipid-like molecule. PKH-26 labeled BAL MVs were intranasally injected into mice and the fluorescence of the bronchial epithelial cells was analyzed. Figure 6a and 6b demonstrate the visible increase in fluorescence of the bronchial epithelial cells isolated from untreated mice and mice that inhaled MVs incubated with PKH-26. When quantified, there was a 60% increase in fluorescence in the lipid-labeled MVs (Figure 6c). The increase in fluorescence demonstrates that BAL MVs are able to transfer their lipid contents to bronchial epithelial cells. Given this, the goal was to analyze the SPH content of tracheal epithelial cells after treatment with BAL MVs in PBD1 mice. Figure 6d, 6e, and 6f depict the fluorescence in sham, PBD1, and BAL MV treated PBD1 mice, respectively. Fluorescence intensity correlates to the SPH content. There is a 35% decrease in SPH in PBD1 mice. However, when treated with MVs, the SPH content is significantly increased from PBD1 (Figure 6E-G) and equivalent to sham levels (Figure 6D, F, G). This demonstrates that BAL MVs can be used to transfer lipid contents, PKH-26 or SPH, to bronchial epithelial cells.

It has previously been demonstrated that SPH has antimicrobial effects^{6,8}. Similarly, we show in Figure 5 that BAL MVs are able to directly kill bacteria. Given that lipid contents of MVs are able to be transferred to bronchial epithelial cells, we sought to determine if we could enrich MVs with SPH to provide enhanced bacterial killing. In Figure 7, we again demonstrate in an *in vitro* assay that BAL MVs result in 25% reduction in bacterial growth. However, when these MVs are incubated with SPH, the SPH enriched MVs have enhanced bacterial killing such that there is a 99% reduction in bacterial growth.

Discussion

Here, we investigated the impact of BAL MVs on *PA* lung infection following scald injury in a murine model. Our initial observation was that the quantity of BAL MVs decreased in scald injured mice. The burn injured mice, with reduced BAL MVs due to injury, have increased mortality associated with development of severe pulmonary infection. However,

restoration of MVs following injury mitigates pulmonary infection and improves survival in susceptible burn-injured mice. Additionally, cytokine analysis demonstrates that MV administration is able to restore IL-6 levels to sham levels. Not only are the antimicrobial effects observed in a burn pneumonia model, but we demonstrate that antimicrobial properties of MVs can be applied to other injury models. Using CLP as a murine model of intra-abdominal sepsis, we demonstrate that local administration of MVs results in improved bacterial clearance locally within the peritoneum as well as systemically in the blood. Finally, we provide two mechanisms by which BAL MVs improve bacterial clearance and reduce mortality. First, BAL MVs directly kill bacteria as demonstrated by *in vitro* assays. Second, MVs act as a SPH donor, a lipid known to have antimicrobial activity. Altogether, these data suggest that BAL MPs provide an innate defense mechanism against bacterial pathogens in healthy individuals, but these levels are depleted following injury. However, MV restoration after injury is able to mitigate pulmonary infection and improve survival through direct bacterial killing and SPH lipid transfer, offering a potential therapeutic alternative.

It is known that burn injury produces an inflammatory response^{27–30}. Further, MVs are produced at sites of inflammation and known to modulate the immune response^{14,19,22}. While platelet derived MVs are the most abundant and well researched population²⁰, NDMVs are the predominant population generated at infected and inflamed foci in critically ill patients during sepsis and injury¹⁴. Neutrophils are an essential part of the innate immune system by responding to sites of inflammation so it would follow that NDMVs are the predominant MV population. These MVs have been shown to be released from neutrophils in response to a number of stimuli including bacteria, endotoxin, TNF-alpha, complement, platelet activating factor, and IL-8, all of which are present in sepsis^{31–33}. We previously demonstrated that peritoneal neutrophil-derived MVs (NDMV) were generated after CLP³⁴ and resulted in increased mortality and a counter-inflammatory response^{14,19}. This non-beneficial counter-inflammatory response is thought to be related to expression of phosphatidyl serine (PS), a signal for apoptosis and uptake by macrophages¹⁹. We postulate that increases in PS-expressing NDMVs would cause a similar influence as apoptotic cells during sepsis, increased mortality, decreased pro-inflammatory cytokine production and increased bacterial burden³⁵. Additionally, in an *in vitro* assay, NDMV co-cultured with bacteria, resulted in increased bacterial count.

In direct contrast to the action of NDMVs, we observed that BAL MVs improve survival and bacterial clearance. Previous research on BAL MVs has suggested that this MV population has a role in immunity and acts as a conveyor of signals in allergen and infectious immune response^{36,37}. Supporting their role in immunity was the finding that BAL MVs are shown to express CD36, which has been implicated in bacterial recognition³⁸. Here, BAL MVs are reduced following a murine burn-injury / pulmonary infection model, but restoration of BAL MVs improves survival and bacterial clearance. Unlike NDMVs, our *in vitro* assays demonstrate that BAL and tracheal epithelial MVs result in enhanced bacterial clearance. From this we conclude that the parent cell of BAL MVs is tracheal epithelial cells, rather than neutrophils, due to the similar antimicrobial activity of BAL and tracheal epithelial cell derived MVs. Additionally, these data demonstrate that MPs divergently mediate the response to infection. From this, we conclude that BAL MVs, in contrast to NDMVs, are

able to uniquely enhance bacterial clearance such that survival is improved. Not only did we demonstrate this in a burn / pneumonia model, but these BAL MPs were able to reduce bacteria locally and systemically when injected into the peritoneum after intra-abdominal sepsis in a CLP injury model. These findings have broad implications as it offers a novel therapeutic approach for local infection and systemic sepsis in multiple injury models, but also a cautionary aspect as not all MVs may enhance bacterial clearance.

We suggest two potential mechanisms by which BAL MVs provide antimicrobial activity and improve survival. First, we demonstrate that BAL MVs directly kill *PA*. From this it would follow that reduced MV levels would result in reduced survival and bacterial clearance. We also suggest that a second mechanism of the antimicrobial activity of BAL MVs is due to the SPH content of these MVs. MVs are a known source of the lipid SPH³⁹. Similarly, we confirm that SPH is present in BAL MVs as administration of MVs resulted in increased SPH content of bronchial epithelial cells. It is known that SPH is present in cell membranes and has antimicrobial activity against a variety of gram-positive and gram-negative bacteria^{11,40}. Our lab has recently demonstrated that SPH is pathologically reduced following burn injury, resulting in increased mortality and reduced bacterial clearance⁶. The antimicrobial effects were similarly demonstrated in a cystic fibrosis model⁸. In both models, restoration of SPH abrogated pulmonary infection. Similarly, we show that burn injured mice are more susceptible to pulmonary infection, resulting in increased mortality. Here, we present the novel data that injury-induced reduction of MVs, which inherently contain the antimicrobial lipid SPH, results in reduced survival and bacterial clearance. We demonstrate that restoration of these SPH containing BAL MVs improves *PA* clearance and thereby improves survival. Further, through a gain of function study, we artificially augment the SPH content of BAL MVs and demonstrate enhanced *PA* killing. Importantly, we also demonstrate that the SPH content of MVs is able to be transferred to bronchial epithelial cells where SPH has been shown to be deficient after injury^{6,8}. This supports previous research that restoration of SPH to levels in healthy murine populations is able to abrogate pulmonary *PA* infection^{6,8}. Further, we suggest that these MVs may provide a novel therapeutic approach due to their direct killing effects and delivery of the antimicrobial lipid SPH.

Burn injury results in marked susceptibility to pulmonary *PA* infection. This is thought to be related to the systemic immunosuppressed state following injury^{28–30}. However, the mechanism of increased susceptibility to *PA* remains unclear. Further investigation may provide insight into mechanisms by which SPH and SPH containing BAL MVs provide their therapeutic effects. While we provide the novel data that BAL MVs provide enhanced antimicrobial activity, further research is necessary to elucidate properties and mechanisms by which BAL MVs produce antimicrobial effects while other MV populations, such as neutrophil derived, result in increased bacterial growth. We speculate that this is due to alterations in lipid composition and that the lipid SPH contributes to some antimicrobial effect. Conversely, the presence of phosphatidyl serine in NDMVs is pathogenic¹⁹. Nonetheless, further research is necessary such that these MVs could be engineered for therapeutic effect and lipid delivery. Our data demonstrates that BAL MVs did not act through a neutrophil mediated mechanism as neither neutrophil numbers nor activation were altered with MV administration. Research investigating molecular pathways and signaling

that are involved with BAL MVs may further help to elucidate how this distinct MV population is able to provide unique antimicrobial effects. Further research on enzymes that regulate expression of SPH in MVs and their impact during infection would be beneficial in development of novel therapies.

Conclusions

This research has broad implications in elucidating the role of MVs in innate defense mechanisms against bacterial pathogens. While we focused on a burn / pneumonia model, our data using a CLP suggests that these effects may have far reaching properties in numerous models. Thus, we conclude that SPH-containing MVs can be used to enhance bacterial clearing such that survival is improved. SPH expressing MVs have beneficial effects in the immune response such that a deficiency of this MV population following injury results in increased susceptibility to bacterial infection, while restoration of MV levels is able to protect susceptible populations from infection. Our data may motivate further investigations to identify populations in which restoration of MVs has the potential to reduce morbidity and mortality through this novel antimicrobial approach involving innate immunity. Uniquely, these susceptible patients would have the opportunity to receive MV therapy to prevent pulmonary infection rather than the currently employed post-infection antibiotic treatment strategy.

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Abbreviations

PA	<i>Pseudomonas aeruginosa</i>
SPH	Sphingosine
MV	microvesicle
BAL	Bronchoalveolar Lavage
PBD1	post burn day one
CLP	cecal ligation and puncture
NDMV	neutrophil derived microvesicles

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Figure 1.

Burn injury reduces total number and sphingosine content of bronchoalveolar lavage microvesicles (BAL MVs). (A) BAL MVs were isolated and quantified from sham or post-burn day one mice. (B) sphingosine content was quantified. * $p < 0.05$ vs sham, $n = 5-6$ mice/group. PBD1, post-burn day 1.

Figure 2.

Bronchoalveolar lavage microvesicles (BAL MVs) protect burn-injured mice from pulmonary *Pseudomonas aeruginosa* (PA) infection and decrease bacterial load. BAL MVs from healthy donor mice were intranasally administered to burn-injured mice prior to intranasal inoculation with PA. (A) Sham, post-burn day one (PBD1) untreated, and PBD1 MV treated mice were monitored for survival. *, $p < 0.05$ vs sham, #, $p < 0.05$ vs PBD1; $n = 17$ – 18 mice/group. (B) BAL fluid was collected 4 hours after infection and bacterial colony-forming units were enumerated. * $p < 0.05$ vs sham; # $p < 0.05$ vs PBD1; $n = 7$ – 9 mice/group. MP, macrophage

Figure 3.

IL-6 decreases after bronchoalveolar lavage microvesicles (BAL MV) treatment with no difference in quantity of neutrophils. BAL fluid was collected for cytokine and cellular analysis. (A) IL-6 was quantified for sham, post-burn day one (PBD1), and MV treated PBD1 mice. *, $p < 0.05$ vs sham; $n=8-9$ mice/group. (B) Neutrophils were quantified for sham, PBD1, and MV treated PBD1 mice. No significant difference found between the cohorts. $n=8-9$ mice/group.

Figure 4.

Local and systemic infection decreases with intraperitoneal bronchoalveolar lavage microvesicles (BAL MV) treatment in the cecal ligation and puncture (CLP) model. Peritoneal lavage (local) and blood (systemic) samples were collected 18 hours after intraperitoneal BAL MV treatment. (A) Local and (B) systemic infection decreases with MV treatment. *, $p < 0.05$ vs CLP; $n=10-16$ mice/group. CFU, colony-forming unit

Figure 5.

Divergent killing of *pseudomonas aeruginosa* (PA) by microvesicle populations. MVs isolated from (A) BAL fluid, (B) cultured tracheal epithelial cells, and (C) cultured neutrophils were incubated with PA in an *in vitro* assay and colony-forming units (CFU) enumerated. *, $p < 0.05$ vs saline treated; $n=20$.

Figure 6.

Bronchoalveolar lavage microvesicles (BAL MVs) can transfer lipids to epithelial cells and be used as a sphingosine (SPH) donor. Mice were treated with (A) vehicle BAL MVs or (B) PKH-26-labeled BAL MVs and lungs were fixed for fluorescent analysis. (C) Quantification of fluorescence in arbitrary units (a.u.) between the 2 cohorts. *, $p < 0.05$ vs vehicle; $n=18-24$ mice/group. Lungs were harvested from (D) Sham, (E) post-burn day one (PBD1), and (F) BAL MV treated PBD1 mice and stained with anti-SPH antibodies. (G) Quantification of fluorescence between cohorts. *, $p < 0.05$ vs sham; $n=4$

Figure 7.

Sphingosine (SPH) enriched microvesicles have enhanced bacterial killing. *Pseudomonas aeruginosa* colony-forming units were enumerated after incubation with vehicle, Bronchoalveolar lavage microvesicles, and SPH enriched macrophages (MP). * $p < 0.05$ vs vehicle; # $p < 0.05$ vs control MV; $n=8$. CFU, colony-forming unit