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GM-1111 PRESERVES PHAGOCYTIC FUNCTION OF MACROPHAGES EXPOSED TO PROLONGED HYPEROXIA VIA INTERRUPTION OF HMGB1-MEDIATED SIGNALING

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GM-1111 PRESERVES PHAGOCYTIC FUNCTION OF MACROPHAGES EXPOSED TO PROLONGED HYPEROXIA VIA INTERRUPTION OF HMGB1-MEDIATED SIGNALING

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ABSTRACT

GM-1111 PRESERVES PHAGOCYTIC FUNCTION OF MACROPHAGES EXPOSED TO PROLONGED HYPEROXIA VIA INTERRUPTION OF HMGB1-MEDIATED SIGNALING

Lee-Anne Daley

Supraphysiological levels of oxygen (i.e. hyperoxia) are used to treat patients with respiratory distress. Prolonged exposure to hyperoxia can impair alveolar macrophage functions and increase susceptibility to ventilator-associated pneumonia (VAP). Hyperoxia-induced alveolar macrophage dysfunction is, in part, mediated by high airway levels of the pro-inflammatory mediator, high mobility group box-1 (HMGB1). An early generation glycosaminoglycan (GAG), 2-O, 3-O desulfated heparin (ODSH), attenuates hyperoxia-compromised innate immunity by preventing the binding of HMGB1 with receptors that activate pro-inflammatory pathways. In this study, we investigated whether the next generation GAG, GM-1111, can attenuate hyperoxia-compromised macrophage function. GM-1111 (100μM) prevented hyperoxia-induced (95% O2 for 24 h) dysfunction of phagocytosis in RAW 264.7 macrophages GM-1111 (0.1-100μM) had no significant effect on the extracellular accumulation of HMGB1 in cultured macrophages produced by hyperoxia. GM-1111 significantly decreased HMGB1-mediated phagocytic dysfunction in RAW 264.7 cells. Localized surface plasmon

resonance data indicated that GM-1111 had a high binding affinity (KD = 3.77x10-8M) to HMGB1. GM-1111 also significantly decreased NF-κB/AP-1 activation and the extracellular accumulation of TNF-α from hyperoxia-compromised macrophages. Overall, our results indicate that GM-1111 attenuates hyperoxia-compromised macrophage function by inhibiting HMGB1-mediated impairment of macrophage phagocytosis and downstream pro-inflammatory responses. Thus, GM-1111 may serve as a potential novel treatment for VAP.

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Introduction:

Mechanical ventilation (MV) and noninvasive oxygen therapy are life-saving interventions often used to treat trauma patients, pre-term neonates and individuals in respiratory distress (Koenig and Truwit, 2006; Vincent *et al.*, 2010; McGrath and Asmar, 2011). High-flow nasal cannulas are one non-invasive method of oxygen supplementation with a higher patient comfort level than conventional oxygen therapy which has been shown to reduce the rate of intubation (Schwabbauer *et al.*, 2014; Zhao *et al.*, 2017). Noninvasive oxygen delivery is becoming more common and use in patients experiencing respiratory failure has increased 30% (Hill, 2013). During oxygen therapy, patients are supplied with supraphysiological levels of oxygen (25-99%) also known as hyperoxia (Kennedy and Nelson, 2013). Although invasive MV and noninvasive means of oxygen therapy can improve patient outcomes, they may also adversely increase patients' susceptibility to respiratory infections known as hospital-acquired (HAP) and ventilator associated pneumonia (VAP). HAP is pneumonia occurring 48 hours or more after hospital admission and accounts for 25% of all ICU infections (American Thoracic Society and Infectious Diseases Society of America, 2005). VAP occurs within 48-72 hours of MV in 9-27% of patients and accounts for approximately 86 % of nosocomial pneumonias with an incidence rate of 5-10 per thousand hospital admissions (American Thoracic Society and Infectious Diseases

Society of America, 2005; Koenig and Truwit, 2006; Heredia-Rodríguez *et al.*, 2016; Timsit *et al.*, 2017). In addition, these oxygen therapy associated conditions can increase the duration of hospital stays and cost of care by 40,000 USD (Rello *et al.*, 2002; American Thoracic Society and Infectious Diseases Society of America, 2005; Six *et al.*, 2016; Timsit *et al.*, 2017). Current treatment for these pulmonary infections involves prophylactic measures, palliative care and broad-spectrum antibiotics, but with the rise of multidrug resistant strains of bacteria and the lack of new antibiotics being developed, other novel therapies are urgently needed (American Thoracic Society and Infectious Diseases Society of America, 2005; Vincent *et al.*, 2010; Kalanuria *et al.*, 2014; Timsit *et al.*, 2017).

Alveolar macrophages are the first line of defense against invading pathogens in the lung (Nicod, 2005; Pinkerton *et al.*, 2014). Our lab and others have demonstrated that prolonged exposure to hyperoxia induces oxidative stress which can impair the ability of alveolar macrophages to phagocytose and kill bacteria (O'Reilly *et al.*, 2003; Morrow *et al.*, 2007; Patel *et al.*, 2016). Furthermore, we have shown in laboratory models simulating VAP in mice and in cell culture, that the presence of the potent DAMP molecule, high-mobility group box-1 (HMGB1), mediates VAP pathogenesis in part by inducing alveolar macrophage dysfunction (Patel *et al.*, 2013, 2016; Entezari *et al.*, 2014; Sitapara *et al.*, 2014; Wang *et al.*,

2015). HMGB1's role in macrophage function has also been demonstrated by Zettel and colleagues, who found that increased levels of HMGB1 in culture media led to decreased clearance of *E. coli* by murine peritoneal macrophages as well as human monocyte-derived macrophages (Zettel *et al.*, 2017). Moreover, VAP patients with lung infections have significantly higher levels of HMGB1 (17.4 ng/mL) in the airways when compared to healthy volunteers (1.7 ng/mL) (van Zoelen *et al.*, 2008). HMGB1 is a nuclear protein that can be actively or passively released from cells into the extracellular (Stros, 2010; Yang *et al.*, 2014; Wang *et al.*, 2019). HMGB1 becomes acetylated after which, it translocates from the nucleus to the cytoplasm, eventually leaving the cell (Yang *et al.*, 2014; Wang *et al.*, 2019). Once extracellular, HMGB1 acts as a ligand to receptors such as RAGE and promotes the release of proinflammatory cytokines, driving an inflammatory cascade which results in cellular damage and dysfunction (Wang *et al.*, 2004; Lu, Wang, *et al.*, 2014; Wang *et al.*, 2019; Paudel *et al.*, 2019).

Extracellular HMGB1's proinflammatory activities have been shown to promote lung injury and exacerbate the pathogenesis of VAP. This has been demonstrated through the use of HMGB1 antibodies ameliorating bacterial load, markers of lung injury, leukocyte infiltration, and cytokine (Patel *et al.*, 2013; Ming *et al.*, 2016; Liming *et al.*, 2018). Our group has found that mitigating the release and extracellular accumulation of

HMGB1 from macrophages can attenuate hyperoxia-compromised macrophage functions (Patel *et al.*, 2013, 2016; Entezari *et al.*, 2014; Sitapara *et al.*, 2014; Wang *et al.*, 2015).

Currently, prophylactic measures of VAP prevention include patient hygiene, sterility of tools used for intubation and administration of antibiotics. With the rise of multidrug resistant bacteria, it is important to find other avenues of improving patient outcomes with modulation of inflammatory mediators. Glycosaminoglycans (GAG) are sulphated or non-sulphated chains of polysaccharides often found in the upper airway and extracellular matrix (Souza-Fernandes *et al.*, 2006; Taylor and Gallo, 2006). One notable GAG, ODSH (2-O, 3-O Desulfated Heparin), is a modified non-coagulant heparin that has been shown to reduce extracellular HMGB1 accumulation and its interaction with cell surface receptors (TLR2 and TLR4) responsible for innate immune activation (Griffin *et al.*, 2014; Sharma *et al.*, 2014; Zheng *et al.*, 2017). Despite ODSH's remarkable therapeutic potential, its clinical use was hampered by the limited sustainability of ODSH derived from animal sources (porcine) and susceptibility to disease contamination and population fluctuations (Jeske *et al.*, 2019; Kouta *et al.*, 2019; Vilanova *et al.*, 2019). Thus, new treatments are urgently needed to mitigate the adverse morbidities associated with mechanical ventilation. A class of sulphated GAG derived from hyaluronic acid (HA) in lieu of animal intestine may be

more accessible and sustainable for global clinical treatment. HA is also abundant in the extracellular matrix of the lung (Souza-Fernandes *et al.*, 2006). Multiple studies have shown the efficiency of HA in attenuating severe inflammation and injury as judged by a reduction of histological scores, secretion of pro-inflammatory cytokines (TNF-α and IL-1β), and infiltration of macrophages and eosinophils (Johnson *et al.*, 2018; Shi *et al.*, 2019). Furthermore, in a mouse model of LPS-induced ALI, pretreatment with HA reduced production of inflammatory cytokines (such as TNF-α, IL-6 and INF-β) and attenuated the airway excessive infiltration of leukocytes (Xu *et al.*, 2015). While high molecular weight (HMW) hyaluronans have been shown to mitigate inflammation, they are prone to degradation into smaller fragments exhibiting proinflammatory properties (Turino and Cantor, 2003; Jiang *et al.*, 2005; Xu *et al.*, 2015; Johnson *et al.*, 2018). Thus, HA was structurally modified to generate a class of stable hyaluronan, GM-1111, which has been shown to interfere with HMGB1 binding to cell surface receptors responsible for macrophage activation (e.g., TLR4) or pyroptosis (RAGE) (Zhang *et al.*, 2011; Alt *et al.*, 2018). GM-1111's ability to inhibit binding of HMGB1 to RAGE may play an important role in mitigating inflammation as demonstrated by it having efficacious effects in murine models of bladder cystitis, rhinosinusitis, and rosacea (Oottamasathien *et al.*, 2011; Zhang *et al.*, 2011; Alt *et al.*, 2018). However, it is unknown whether GM-1111

plays a protective role in lung diseases characterized in part by inflammation such as VAP. Thus, the purpose of this study was to determine if GM-1111 could ameliorate hyperoxia-compromised macrophage functions mediated by HMGB1 and other comorbidities occurring in VAP.

Methods and Materials:

1.Cell Culture and Reagents

Murine macrophage-like RAW 264.7 cells (ATCC TIB-71, American Type Culture Collection, Manassas, VA, USA) were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine (American Type Culture Collection (ATCC) 30-2002, Manassas, VA, USA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA, USA). RAW-Blue cells (RAW 264.7 macrophages with a chromosomal integration of NF-kB/AP-1 – induced secreted embryonic alkaline phosphatase) reporter gene (raw-sp, InvivoGen, San Diego, CA, USA) were cultured in high glucose DMEM with L-glutamine and 10% heat-inactivated fetal bovine serum supplemented with Normocin (InvivoGen, San Diego, CA, USA, 50mg/mL) and Zeocin (InvivoGen, San Diego, CA, USA, 100 mg/mL) Cells were maintained at 37°C and 5% CO_2 and subcultured after reaching 80-90% confluency. Cells were subcultured by scraping, placed in a falcon tube and centrifuged at 850 RPM for 5 minutes at 4˚C, and counted using a Countess II FL automated

Cell Counter (ThermoFisher Scientific, Waltham, MA, USA). For all experiments, cells were allowed to adhere for 4-6 hours, followed by exposure to room air (21% O_2) or hyperoxia (95% O_2) in the presence or absence of GM-1111 (diluted in minute amounts of PBS) (Glycomira, Salt Lake City, UT, USA) (0.1 -100 µM) for 24 hours. Hyperoxic exposure was performed in sealed, humidified plexiglass chambers (Billups-Rothenberg Inc., DEL Mar, CA, USA) that were flushed with 95% oxygen. The MiniOX oxygen analyzer (MSA, Medical Products, Pittsburgh, PA, USA) was used to monitor O_2 levels. Cells were also exposed to recombinant HMGB1 at normoxic conditions in the presence or absence of GM-1111.

2. Phagocytosis Assay

RAW 264.7 cells were seeded in 24-well plates, allowed to adhere and incubated with GM-1111 (0.1 -100 µM), in the presence or absence of 10 ug/mL of recombinant HMGB1 (donated by Dr. Haichao Wang of The Feinstein Institute of Medical Research, Manhasset, NY) and exposed to hyperoxia for 24 hours. Subsequently, the cells were incubated at 37°C for 1 hour with fluorescein isothiocyanate (FITC)- labeled minibeads (Polysciences, Warrington, PA, USA) opsonized in fetal bovine serum (FBS), at a ratio of 100:1 beads per cell. Cells were immediately placed on ice and washed 3 times with ice-cold PBS to stop phagocytosis. Cells were then fixed using 3.7% paraformaldehyde (#J61984, Alfa Aesar, Ward Hill, MA, USA) Nuclei were stained with spell out DAPI (DAPI, Sigma-Aldrich,

St. Louis, MO, USA), and the cytoplasm was stained with rhodamine phalloidin (#PHDR1 Cytoskeleton, Inc. Denver, CO, USA). Cells were imaged using the EVOS FL Auto Imaging System (ThermoFisher Scientific, Waltham, MA, USA). Phagocytic activity was assessed by quantification of FITC minibeads per cell for approximately 200 cells per cell using the ImageJ Software (National Institutes of Health, Bethseda, MD, USA). The phagocytotic activity was reported as a percent relative to control groups (room air for hyperoxic studies and 0 μg/mL of GM-1111 in HMGB1 studies).

3. NF-κB/AP-1 Activity

NF-κB/AP-1 activity was determined using RAW-Blue cells following the manufacturer's instructions. Cells were incubated with LPS (1 μg/mL) (LPS-RS, # tlrl-rslps, Invivogen, San Diego, CA, USA) and GM-1111 (0.1 - 100 μM) for 24 hours. The absorbance was measured using a FilterMax F5 Multi-Mode Microplate Reader (Molecular Devices).

4.HMGB1 Release

Raw 264.7 cells were seeded in 6-well plates and allowed to adhere. Cells were then placed in 21% O_2 or 95% O_2 in the presence or absence of GM-1111 prepared in reduced-serum Opti-MEM media (Gibco/BRL Life Technologies Inc., Grand Island, NY) for 24 hours. The cell culture media was collected and concentrated in Centricons (Merck Millipore Ltd., Carrigtwohill, IRL).

5. Western Blot Analysis

RAW 264.7 cells were seeded in 6-well plates and allowed to adhere. Cells were then placed in 21% O_2 or 95% O_2 in the presence or absence of GM-1111 prepared in reduced-serum Opti-MEM media (Gibco/BRL Life Technologies Inc., Grand Island, NY) for 24 hours. Subsequently, the media was removed, and plates were washed 3x with PBS. Two hundred µL of Cell Lysis Buffer (Cell Signaling technologies, Danvers, MA, USA), 1% phenylmethylsulfonyl fluoride (PMSF, Sigma Aldrich, St.Louis, MO, USA),and 1% protease inhibitor cocktail (Sigma Aldrich, St.Louis, MO, USA) was added and plates were shaken, at 4°C for 15 minutes. Cell lysates were scraped by hand, sonicated, and then centrifuged at 10,000 RPM for 10 minutes at 4˚C. Protein concentration of cell lysates was determined using the Pierce bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL, USA). 10% of concentrated supernatants were loaded onto a 12% SDS-PAGE gel (BioRad Laboratories, Hercules, CA, USA) and then transferred to polyvinylidene fluoride membranes (Millipore, Burlington, MA, USA). Nonspecific binding sites were blocked by adding Pierce Clear Milk Blocking Buffer (Pierce, Rockford, IL, USA) diluted in Tris-buffered saline, containing 1% Tween 20 (TBST), and incubating for 1hr at room temperature. The membranes were washed three times with TBST and incubated overnight at 4°C with anti-HMGB1 rabbit antibody (1:1000 Sigma-Aldrich, St.Louis, MO, USA). The

membranes were washed with TBST and incubated for 1 hour with anti-rabbit (1:1000) horseradish peroxidase-coupled secondary antibody (GE Healthcare, Little Chalfont, Buckinghamshore, UK). The membranes were washed again 3 times with TBST, and proteins were visualized using the Super Signal West Pico chemiluminescent substrate kit (ThermoFisher Scientific, Waltham, MA, USA). Images were developed using the ChemiDOC MP Imaging System (BioRad) and bands were quantified using ImageJ Software (National Institutes of Health, Bethseda, MD, USA).

6. ELISA Assay

TNF-α levels in cell culture supernatant, which were obtained as previously described, were determined using the TNF-alpha Mouse Uncoated Elisa Kit (CAT # 88732422, Invitrogen, Waltham, MA, USA), according to manufacturer's instructions. The absorbance was measured at 535 nm using Biotek Synergy LX Multimode Reader (Biotek, Winooski, VT, USA). The values obtained from this were converted to indicate the percent TNF release compared to the hyperoxia control group.

7. MTT Assay

To assess cell viability, the MTT assay was performed. RAW 264.7 cells were seeded in 48-well plates, allowed to adhere and treated with GM-1111 in the presence or absence of hyperoxia for 24 hours. 0.5 mg/mL of MTT (EMD Millipore Corp, Burlington, MA, USA) in DMEM without L-glutamine and phenol red (17-205-CV, Corning, Manassas, VA, USA) was

added to the plate which was left to incubate for 2 hours at 37°C. MTT reagent was removed and isopropyl alcohol was added to the plates to solubilize formazan crystals. After ten minutes the liquid in the wells was mixed and 200µL from each plate were transferred to a new 96-well plate. The plate was read on the Multiskan EX spectrometer (Thermofisher Scientific, Waltham, MA, USA) at 570 nm.

8. Statistical Analysis

The data are presented as the standard error of the mean (SEM) of at least three experiments. The data were analyzed using Student's t test or ANOVA. The *post hoc* analyses were conducted using Dunnet's test. The *a priori* significance level was $p < 0.05$.

FIGURES

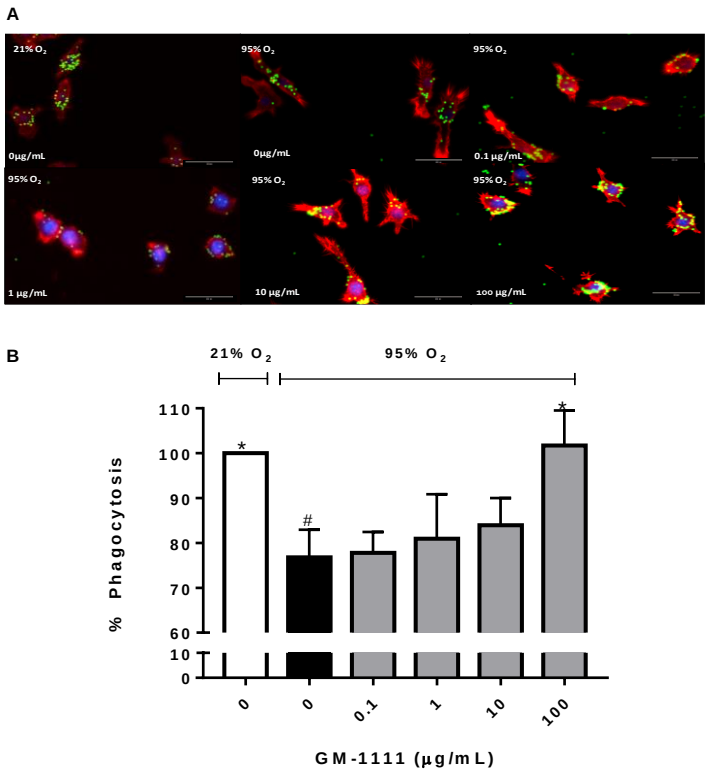

Figure 1.

Figure 1. FR. RAW 264.7 cells were exposed to room air (21% O_2) or 95% O_2 and vehicle or GM-1111 (0.1, 1, 10 or 100 ug/mL) for 24 hours. Cells were subsequently incubated with FITC-labeled latex minibeads (green) for 1 hour and stained with DAPI and rhodamine phalloidin to visualize the nuclei (blue) and actin (red) cytoskeleton respectively. (*A*) Shows representative immunofluorescence images of RAW 264.7 cells. (*B*) The bar graph represents the percent phagocytosis that was determined by counting beads per cell for at least 200 cells per treatment group. Each value represents the mean ± SEM of three independent experiments for each group. #$p < 0.05$, compared to cells exposed to 21% oxygen. *$p < 0.05$, compared with cells exposed to 95% O_2 control.

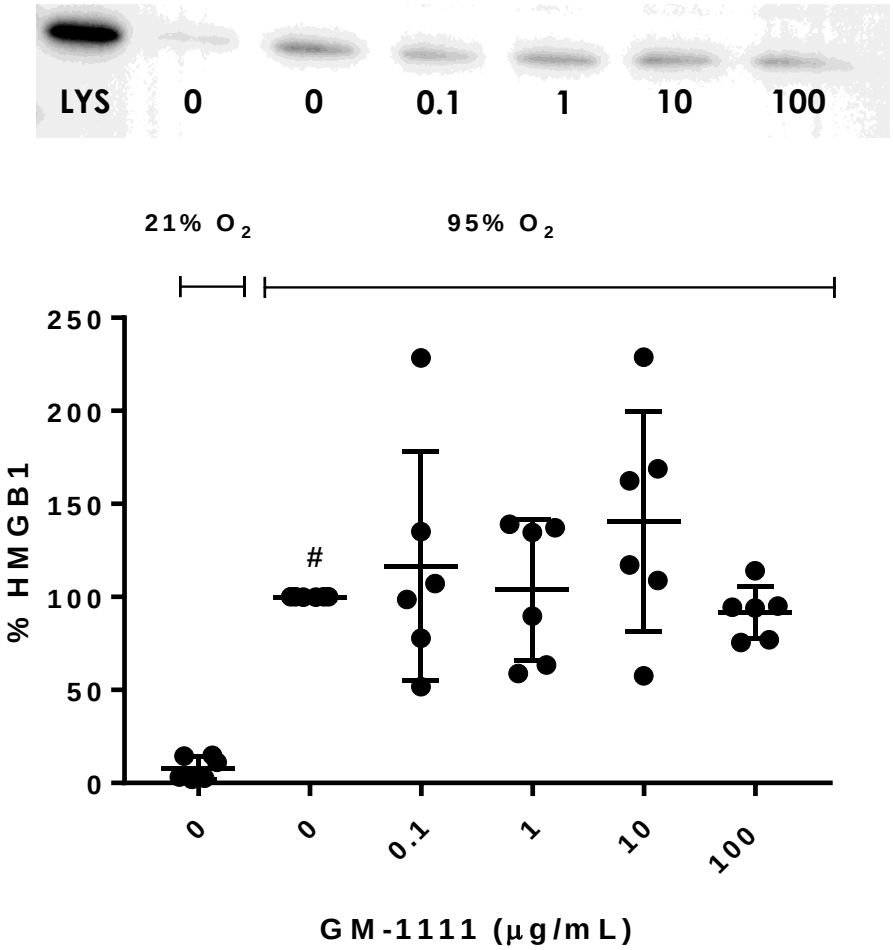

Figure 2.

Figure 2. GM-1111 does not significantly attenuate hyperoxia-induced accumulation of extracellular HMGB1. RAW 264.7 cells were exposed to room air (21% O_2) or 95% O_2 or GM-1111 (0.1, 1, 10 or 100 ug/mL) for 24 hours. The levels of extracellular HMGB1 in the culture media of RAW 264.7 cells were determined by Western blot analysis. The levels of HMGB1 were quantified by measuring the integrated density value of the immunoreactive bands on Western blots and expressed as percent HMGB1 relative to the 95% O_2 control group. Each value represents the mean ± SEM of three independent experiments. #$p < 0.05$ compared to cells exposed to 21% O_2.

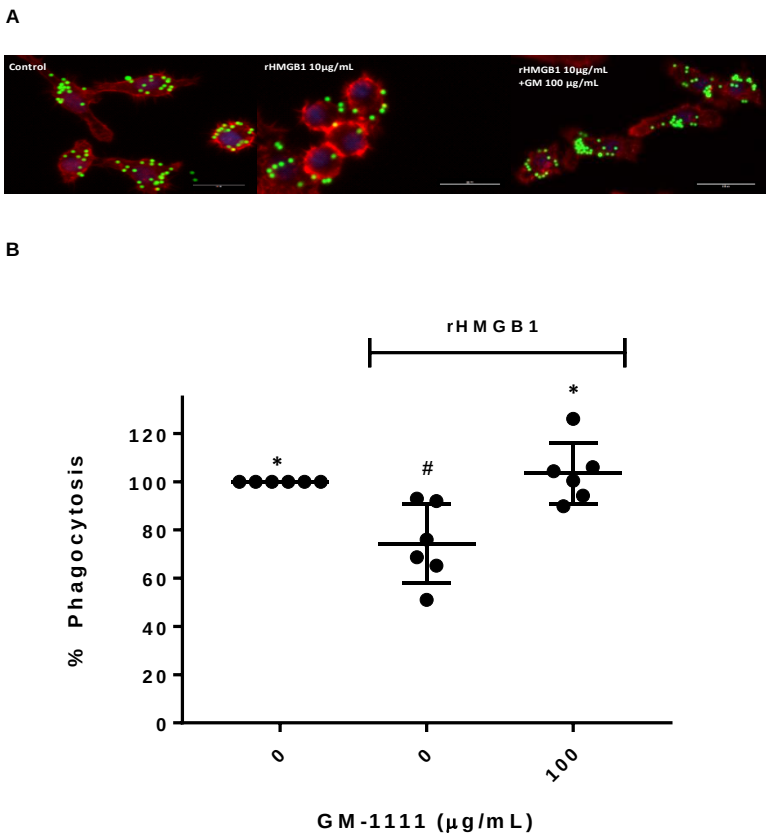

Figure 3.

Figure 3. GM-1111 restores the phagocytic ability of HMGB1- compromised macrophages. RAW 264.7 cells were cultured in media or in the presence of recombinant HMGB1 (10 µg/mL) or 100 µg/mL of GM-1111 for 24 hrs. Cells were subsequently incubated with FITC-labeled latex minibeads (green) for 1 hour and stained with DAPI and rhodamine phalloidin to visualize the nuclei (blue) and actin (red) cytoskeleton respectively. (*A*) Shows representative immunofluorescence images of RAW 264.7 cells. (*B*). The bar graph represents the percent phagocytosis as determined by counting the beads per cell for at least 200 cells per treatment group. Each value represents the mean ± SEM of three independent experiments for each group. #p < 0.05, compared to control group. *p <0.05, compared to recombinant HMGB1 treated control group.

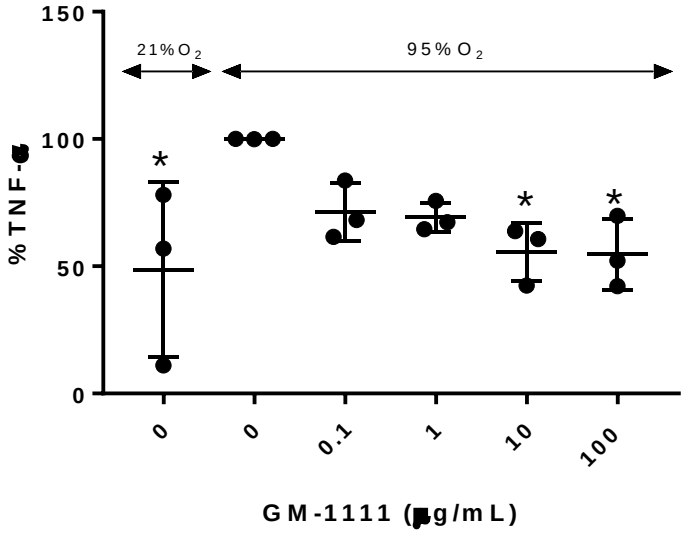

Figure 4. GM-1111 significantly decreases the secretion of TNF-α in macrophages exposed to prolonged hyperoxia. RAW 264.7 cells were exposed to 95% O_2) or GM-1111 (0.1, 1, 10 or 100 ug/mL) for 24 hours. The TNF-α levels in the supernatants were determined using ELISA and reported as a percent relative to the 95% O_2 control group. The graph and values represent the mean ± SEM from three independent experiments. *$p < 0.05$, compared to 95% oxygen control group.

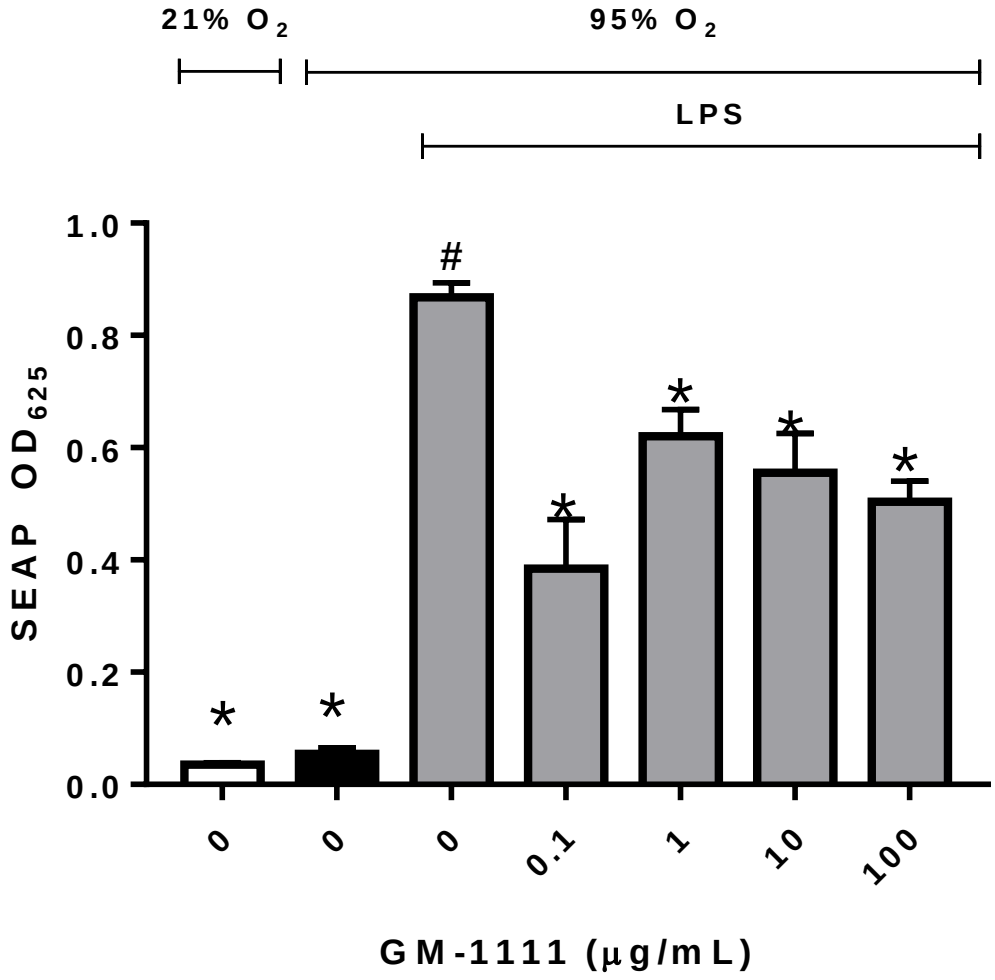

Figure 5. GM-1111 significantly attenuates the activation of NF-κβ/Ap-1 in RAW 264.7 macrophages. The NF-κβ/Ap-1 reporter RAW-Blue macrophages were exposed to room air (21% O_2) or 95% O_2 or LPS (1 μg/mL) and GM-1111 (0.1, 1, 10 and 100 ug/mL) for 24 hours. Cell supernatant was used to determine the level of secreted embryonic alkaline phosphatase (SEAP) reporter using the Quantiblue Assay. The graph and values represent the mean ± SEM from one representative experiment repeated in triplicate. #$p < 0.05$, compared to 21% oxygen group. *$p < 0.05$, compared exposed to 95% O_2 + LPS control group.

Supplementary Figures

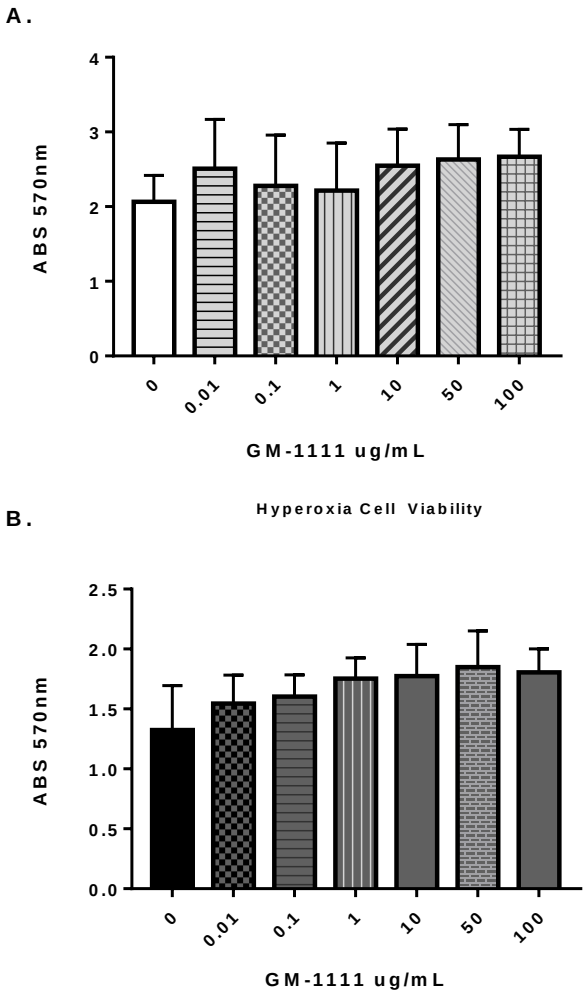

Figure S1 GM-1111 is not cytotoxic to macrophages exposed to Room Air or Hyperoxia

RAW 264.7 cells were exposed to room air (21% O_2) or 95% O_2 and incubated with vehicle or GM-1111 (0.01, 0.1, 1, 10, 50 and 100 ug/mL) for 24 hours. Cell viability was determined using the MTT assay. (A) The bar graph shows the representative values of absorbance at 570 nm for cells cultured in Room Air. (B) The bar graph shows the representative values of absorbance at 570 nm for cells cultured in hyperoxia. The graph and values represent the mean ± SEM from three independent experiments.

Results

1 GM-1111 preserves the phagocytic function of macrophages exposed to hyperoxia

Prolonged exposure to hyperoxia can impair macrophage phagocytosis, resulting in impaired innate immune functions and clearance of gram-negative bacterial airway infections (O'Reilly *et al.*, 2003; Morrow *et al.*, 2007; Patel *et al.*, 2013, 2016; Wang *et al.*, 2015). Previous studies indicate that glycosaminoglycans (GAGs), such as hyaluronic acid, increase bacterial clearance (Håkansson *et al.*, 1980; Ahlgren and Jarstrand, 1984; Liu *et al.*, 2019). Therefore, we determined the effect of the next generation GAG, GM-1111, on hyperoxia-compromised macrophage phagocytic function. As demonstrated in Figure 1B, there was a significant decrease in the phagocytic activity of hyperoxia-exposed macrophages (76.81 ± 3.54%, $p<0.05$) compared to macrophages exposed to normoxic conditions (100 ± 0%). The incubation of RAW 264.7 macrophages with GM-1111, at 0.1 (77.8 ± 2.68%), 1 (80.95 ± 5.72%) or 10 µg/ml (83.97 ± 3.50%) did not significantly increase phagocytic activity, whereas GM-1111, at 100 µg/ml, significantly increased phagocytosis (101.75 ± 4.50%, $p<0.05$) compared to 95% O_2 control. These results indicate that GM-1111 protects macrophages against hyperoxia - mediated phagocytic dysfunction.

2 GM-1111 does not significantly attenuate hyperoxia-induced accumulation of extracellular HMGB1

Previously, we have reported that prolonged exposure to hyperoxia induces the excretion of HMGB1 from macrophages into the extracellular environment and produces hyperoxia-impaired macrophage functions (Patel *et al.*, 2013; Entezari *et al.*, 2014; Wang *et al.*, 2015). Thus, we conducted experiments to determine if GM-1111 affects the accumulation of extracellular HMGB1. As shown Figure 2, macrophages exposed to hyperoxia (100 ± 0.0034%, $p<0.05$) released significantly greater levels of HMGB1 when compared to macrophages that were exposed to normoxic conditions (8.09 ± 2.50%). GM-1111, at 0.1 (116.5 ± 25.17%), 1 (103.8 ± 15.45 %), 10 (140.6 ± 24.18 %), or 100 μg/ml (91.65 ± 5.78 %) had no significant effect on extracellular HMGB1 levels compared to hyperoxic controls. Thus, GM-1111 did not significantly decrease the amount of extracellular HMGB1, suggesting that GM-1111's protective effect on phagocytic functions is due to a different molecular mechanism.

3 GM-1111 protects macrophage phagocytosis that was compromised by recombinant HMGB1 (rHMGB1)

Extracellular HMGB1 bind to receptors, such as TLRs and RAGE, activating downstream pathways, which can impair macrophage phagocytosis (Entezari *et al.*, 2012; Patel *et al.*, 2013; Sharma *et al.*, 2014; Wang *et al.*, 2019). GM-1111 has been shown to inhibit the binding of various ligands

to RAGE (Zhang *et al.*, 2011). Therefore, we determined if GM-1111 affects the impairment of RAW 264.7 cells produced by extracellular HMGB1. As shown in Figure 3B, the phagocytic function of macrophages was significantly decreased following incubation with rHMGB1 (74.32 ± 6.64%, $p < 0.05$) compared to the control group, 100 ± 0%). The incubation of macrophages with 100 ug/mL of GM-1111 significantly enhanced phagocytic activity (103.5 ± 5.16%, $p<0.05$) compared to cells incubated with rHMGB1. These data suggest that GM-1111 can preserve macrophage phagocytosis by decreasing the effect of extracellular HMGB1.

4 GM-1111 significantly decreases the hyperoxia-induced extracellular secretion of TNF-α

Prolonged exposure to hyperoxia can produce lung inflammation and cell injury (Davis *et al.*, 1989; O'Reilly *et al.*, 2003; Perng *et al.*, 2010; Patel *et al.*, 2013; Entezari *et al.*, 2014; Wang *et al.*, 2019). In addition to stimulating HMGB1 release, hyperoxia induces TNF-α expression and secretion from alveolar macrophages as well as in murine lung homogenate (Tsan *et al.*, 1995; Horinouchi *et al.*, 1996). Furthermore, studies have linked HMGB1 signaling to TNF-α secretion in various cells, suggesting that HMGB1 plays a major role in mediating hyperoxia-induced proinflammatory cytokine secretion and lung injury (Abraham *et al.*, 2000; Luan *et al.*, 2010; Perng *et al.*, 2010; Zhang *et al.*, 2017). We

conducted experiments to ascertain if GM-1111 attenuates the secretion of TNF-α from macrophages. Hyperoxic 99.99 ± 0.01%, $p<0.05$) secreted significantly higher levels of TNF compared to macrophages cultured in normoxia 48.66 ± 19.78 ,$p <0.05$). The incubation of hyperoxic macrophages with GM-1111, at 0.1 (71.17 ± 6.55%) and 1 ug/ml (69.2 ± 3.342%) did not significantly decrease TNF-α secretion when compared to cells incubated with hyperoxia (99.99 ± 0.01%; Figure 5). However, 10 (55.65 ± 6.687%) and 100 ug/ml (54.72 ± 8.078%, $p<0.05$) of GM-111 significantly decreased TNF-a secretion elicited by hyperoxia (Figure 5).

5 GM-1111 Reduces NF-κB/AP-1 Activation

The binding of extracellular HMGB1 to cell membrane receptors, such as TLRs and RAGE, induces the downstream activation of NF-κB, inducing the release of proinflammatory cytokines and HMGB1 (Luan *et al.*, 2010; Yang *et al.*, 2010; Sun *et al.*, 2018; Wang *et al.*, 2019). Additionally, GM-1111 and other modified hyaluronans inhibit the binding of HMGB1 to RAGE and TLRs (Zhang *et al.*, 2011; Savage *et al.*, 2016). Therefore, we determined the effect of GM-1111 on the activation of NF-κB. Similar to physiological conditions occurring in infectious models of VAP, cells were exposed to both hyperoxia and LPS to activate NF-κB. Following incubation with LPS (0.87 ± 0.015 AU, $p <0.05$), NF-κB/AP-1 activation was significantly greater in macrophages exposed to normoxic (0.035 ± 0.0021 AU) and hyperoxic controls (0.054 ± 0.0078 AU) GM-1111, at 0.1

(0.384 ± 0.0506 AU), 1 (0.6204 ± 0.0274 AU), 10 (0.556 ± 0.0401AU), and 100 μg/ml (0.504 ± 0.0209 AU, $p<0.05$) significantly attenuated NF-κB/AP-1 activation in macrophages stimulated by both hyperoxia and LPS (Figure 5).

Supplementary Figure- Fig S1

GM-1111 does not reduce cell viability in normoxia or affect hyperoxia induced growth arrest

Before exploring the effect of GM-1111 on macrophage function, we conducted experiments to determine if GM-1111 1) is cytotoxic to macrophages and 2) if it increases the viability of macrophages exposed to hyperoxia. The results indicated that GM-1111 produced a non-significant increase in mitochondrial function under room air conditions, but it did not significantly affect mitochondrial activity (2.063 ± 0.206 vs 2.509 ± 0.4649 vs 2.278 ± 0.393 vs 2.215 ± 0.368 vs 2.549 ± 0.283 vs 2.633 ± 0.268 vs 2.667 ± 0.2121, $p<0.05$)(Figure S1A). Our results are congruent with previous findings indicating that the viability of macrophages is decreased following exposure to hyperoxia compared to macrophages exposed to room air (2.063 ± 0.206 vs 1.323 ± 0.214, $p<0.05$) (Shenberger and Dixon, 1999). As seen in Fig S1B, GM-1111, under hyperoxic conditions, produced a non-significant increase in mitochondrial function but did not rescue the growth arrest of macrophages that occurs during prolonged hyperoxia (1.323 ± 0.214 vs

1.544 ± 0.168 vs 1.604 ± 0.104 vs 1.754 ± 0.0991 vs 1.774 ± 0.153 vs 1.849 ± 0.175 vs 1.806 ± 0.1127, $p<0.05$).

Discussion

Prolonged exposure to hyperoxia can compromise host innate immune functions to clear bacterial infections (O'Reilly *et al.*, 2003; Tateda *et al.*, 2003; Morrow *et al.*, 2007; Patel *et al.*, 2013). HMGB1 has been implicated as a key mediator of pathogenesis in VAP by inducing inflammatory lung injury and impairing host defense against bacterial infection. In this study, we have shown that treatment with GM-1111 results in protection against hyperoxia-mediated impairment of macrophage phagocytic function. Interestingly, GM-1111 did not attenuate hyperoxia- induced accumulation of extracellular HMGB1. However, GM-1111 was able to preserve phagocytic function of HMGB1-compromised macrophages. Moreover, GM-1111 significantly reduced hyperoxia-induced TNF-α secretion which is mediated by HMGB1 as well as NF-κβ activity in LPS/hyperoxia treated macrophages. These results suggest that GM-1111 protects macrophage function by inhibiting HMGB1 mediated downstream events and reducing proinflammatory cytokine secretion.

GM-1111 Preserves Macrophage Phagocytic Function

In this study, we demonstrate that treatment with 100 μg/ml GM-1111 is able to ameliorate hyperoxia compromised phagocytosis (Figure 1). Upon

treatment with hyperoxia, macrophage phagocytosis was significantly impaired, and this compromised function was attenuated in macrophages treated with 100 μg/ml of GM-1111. These findings are consistent with other studies in which hyaluronic acid improved bacterial clearance (Håkansson *et al.*, 1980; Ahlgren and Jarstrand, 1984; Liu *et al.*, 2019). In one study, thirty minutes of preincubation of isolated neutrophils from human blood with HA (10-500 μg/l) showed a significant increase in the rate of phagocytosis of immunoglobulin coated latex particles (Håkansson *et al.*, 1980). In the same study, subcutaneous injection of 10 mg of HA into healthy patients resulted in an increased phagocytic rate that remained for 6 days (Håkansson *et al.*, 1980). In another study, thirty-minute preincubation of isolated human monocytes with high molecular weight hyaluronic acid (5-150 μg/ml) stimulated the uptake of opsinized yeast particles (Ahlgren and Jarstrand, 1984). Both studies involve the preincubation of cells with HA and show a stimulation of phagocytosis but differ in the cells examined. Unlike these studies, our study uses a longer incubation time and introduces a stimulus that compromises the cells ability to uptake bacteria. While enhanced phagocytosis is demonstrated in these studies at lower doses, the cells are in a state of unencumbered ability to clear bacteria. Our study examines the clinical condition of VAP and the compromised immune defense that results from prolonged hyperoxia. Our group also introduces

a novel concept of HA not only enhancing the natural immune response, but instead combatting circumstances where the immune response is impaired. To our knowledge, few studies have explored HA's role in rescuing the function of injured or compromised immune cells. In one recent study using ex vivo human lungs, administration of high molecular weight HA (1mg) was able to significantly reduce the levels of E. coli CFU in BALF (Liu *et al.*, 2019). The same HA treatment improved the phagocytosis of LPS-injured human blood monocytes (Liu *et al.*, 2019). LPS is able to induce oxidative stress, inflammation and cell injury through its binding to cellular receptors which causes release of HMGB1 (Wu *et al.*, 2012; Yang *et al.*, 2014; Xu *et al.*, 2015; Jiang *et al.*, 2018). These studies suggest that treatment with HA and similarly structured molecules can enhance phagocytosis of bacteria and that HA may have protective or restorative effects on the function of compromised immune cells.

GM-1111 does not have significant effect on Accumulation of HMGB1

HMGB1 plays a major role in phagocytic dysfunction and it has been shown that as amounts of extracellular HMGB1 increase, there is augmented severity of compromised phagocytic ability (Wang *et al.*, 2004; Friggeri *et al.*, 2010; Entezari *et al.*, 2012). Hyperoxia exposure increases the accumulation of HMGB1 in the airways and induces its release from macrophages. HMGB1, a nuclear binding protein, can be

actively released from innate immune cells upon stimulation from bacterial endotoxin (LPS), proinflammatory cytokines or other injurious stimuli (Wang *et al.*, 2004). Active release of HMGB1 can occur due to post translational modifications such as acetylation, phosphorylation or methylation which induce translocation from the nucleus to the cytoplasm, and subsequently out of the cell (Wang *et al.*, 2004; Lu, Antoine, *et al.*, 2014; Yang *et al.*, 2014). Once in the extracellular milieu, HMGB1 can accumulate *and interact* with extracellular membrane receptors such as RAGE and TLR4 (Wang *et al.*, 2004; Yang *et al.*, 2010; Deng *et al.*, 2013). Ligation of HMGB1 to these receptors initiates downstream events which mediate chemotaxis of neutrophils and macrophages as well as the release of proinflammatory cytokines potentiating a cycle of lung injury and inflammation (Wang *et al.*, 2004; Yang *et al.*, 2010; Deng *et al.*, 2013). Our lab has previously found attenuation of extracellular HMGB1 levels to correlate with increased phagocytic function of RAW 264.7 cells (Patel *et al.*, 2013, 2016; Wang *et al.*, 2015). The deleterious effects of extracellular HMGB1 were mitigated via inhibition of acetylation, translocation and release (Wang *et al.*, 2015; Patel *et al.*, 2016; Zheng *et al.*, 2017). These studies confirm the ability of extracellular HMGB1 to directly compromise macrophage function in a dose dependent manner. Interestingly, our data indicates that the levels of extracellular HMGB1 in GM-1111 treated groups remained as high as

hyperoxic controls (Figure 2). This is noteworthy, as it demonstrates that GM-1111 is able to preserve macrophage function via a different mechanism without attenuating extracellular HMGB1 accumulation. So far there are no studies that examine the role of HA on HMGB1 acetylation. In the future, it would be interesting to investigate whether treatment with GM-1111 affects post translational modifications of HMGB1.

GM-1111 Blocks Extracellular HMGB1-Mediated Phagocytic Dysfunction

As shown in Figure 3, GM-1111 can preserve the phagocytic function of macrophages exposed to recombinant HMGB1. Extracellular HMGB1 has been implicated in the pathogenesis of various diseases such as CF and VAP (Wang *et al.*, 2019). HMGB1 has been found to directly compromise macrophage function as demonstrated through mechanism and functional studies (Entezari *et al.*, 2012). Macrophages treated with recombinant HMGB1 (rHMGB1) (up 1000 ng/ml) exhibited significantly reduced phagocytic activity (Entezari *et al.*, 2012). Administration of neutralizing HMGB1 antibody markedly reduced CFU loads of P. aeuriginosa in the lungs of mice with cystic fibrosis and improved the phagocytic activity of alveolar macrophages as well as peritoneal macrophages (Entezari *et al.*, 2012). Thus, blocking the effects of extracellular HMGB1 on phagocytosis is important to improving innate immunity. Our study used similar means to determine phagocytic ability

but utilized a higher molecular ratio of rHMGB1 to confirm the mechanism by which GM-1111 restores function.

RECEPTORS INVOLVED IN PHAGOCYTOSIS

Toll-like Receptor 4 has been implicated to have a major role in phagocytosis. Anand and colleagues found that treatment with LPS, a TLR4 agonist, increased phagocytosis in J774 macrophages as well as peritoneal macrophages from wild type mice (Anand *et al.*, 2007). Peritoneal lavage from wild type mice also demonstrated significantly higher bacterial yield compared to that of TLR4 mutant mice (Anand *et al.*, 2007). This correlates with other studies showing that treatment with LPS or other TLR4 agonists resulted in increased TLR4 expression, phagocytosis and bacterial killing of M. tuberculosis, Y. pestis or S. pneumoniae in microglia, RAW 264.7 cells, mouse peritoneal cells and THP-1 monocytes (Jain *et al.*, 2008; Ribes *et al.*, 2010; Lv *et al.*, 2017). Interestingly, blockage of the TLR4 receptor with blocking antibody HTA125 resulted in decreased LPS- enhanced rates of phagocytosis (Lv *et al.*, 2017).

HMGB1 has been shown to bind to TLR4 and initiate downstream action resulting in inflammation and phagocytic dysfunction (Yang *et al.*, 2010; Entezari *et al.*, 2012; Deng *et al.*, 2013; Wang *et al.*, 2019). The significance of TLR4 in mediating hyperoxia induced macrophage dysfunction was illustrated when macrophages with dampened TLR4

signaling did not demonstrate compromised phagocytosis (Entezari *et al.*, 2012). In this study, peritoneal macrophages from wild-type mice or mice deficient in TLR2 or TLR4 receptors were grown and treated with healthy BAL and CF patient BAL which contained high levels of extracellular HMGB1 (Entezari *et al.*, 2012). After this treatment, macrophages were exposed to GFP expressing P. aeruginosa and internalized PAO1 were measured per cell (Entezari *et al.*, 2012). Wild type macrophages treated with BAL from CF patients demonstrated significantly impaired phagocytosis compared to those treated with BAL from healthy patients (Entezari *et al.*, 2012). TLR2-deficient macrophages showed higher phagocytic rates compared to wild-type, but the number of bacteria internalized per cell was still significantly lower than that of TLR2-deficient macrophages treated with healthy BAL. However, TLR4 deficient macrophages showed no significant difference in phagocytosis between BAL treatments. This suggests that TLR4 is essential to mediating HMGB1 induced phagocytic dysfunction. While TLR4 is crucial to phagocytosis and its interactions with HMGB1 are tied to dysfunction, it is unclear if and how GM-1111 interacts with the receptor to enhance phagocytic function in conditions of hyperoxia. GM-1111 may solely act by reducing HMGB1's binding affinity to a degree in which phagocytosis is restored or it may also bind to TLR4 directly to compete with HMGB1.

Previous studies have shown that glycosaminoglycans (GAGs) such as hyaluronic acid can improve bacterial clearance via an undetermined mechanism, though there is speculation that interactions occur with cell surface receptors resulting in activation of receptors involved in phagocytosis or enhanced cell metabolism (Håkansson *et al.*, 1980; Ahlgren and Jarstrand, 1984; Liu *et al.*, 2019). Hyaluronan has the ability to signal through TLRs and act as a pattern associated molecular pattern (Termeer *et al.*, 2002; Chang *et al.*, 2007; NOBLE *et al.*, 2011). LMW HA and hyaluronan fragments can induce the secretion of chemokines/cytokines and are markers of cell injury (Termeer *et al.*, 2002; Yamawaki *et al.*, 2009; NOBLE *et al.*, 2011). LMW HA, through a TLR4 mediated process, induces the production of TNF-α, stimulating the maturation and activation of dendritic cells (Termeer *et al.*, 2002). HMW HA demonstrates the ability to inhibit osteoclast differentiation from bone marrow derived macrophages (Chang *et al.*, 2007). This anti-osteogenic activity was determined to be TLR4 dependent as the effect was diminished upon treatment with a TLR4 antibody (Chang *et al.*, 2007). As both HMW and LMW HA interact with TLR4, it is likely that GM-1111 is also able to bind, affecting phagocytosis and downstream events.

GM-1111 Suppresses HMGB1- Facilitated Release of Cytokines and Inhibits HMGB1 Signaling

Hyperoxia has been shown to induce the production of TNF-α mRNA and secretion of TNF-α protein in alveolar macrophages and murine lungs (Tsan *et al.*, 1995; Horinouchi *et al.*, 1996; Nagato *et al.*, 2012). TNF-α, a potent cytokine and early inflammatory mediator, has been linked to inducing oxidative stress and inflammation as well as lung edema, immune cell chemotaxis and the pathogenesis of various inflammatory diseases (Tsan *et al.*, 1995; Schwabe and Brenner, 2006; Malaviya *et al.*, 2017). In our study, we observed significant TNF-α secretion upon 24 hours of hyperoxia treatment (data not shown) that was significantly reduced with GM-1111 treatment (Figure 4). This attenuation supports our hypothesis of GM-1111 interrupting downstream signaling of HMGB1. Reduction of TNF-α secretion was dose dependent with only the higher doses showing significant effect. These results correlate with other studies exploring GM-1111's efficacy in reducing the production and release of inflammatory cytokines such as IL-8, TNF-α, IL-1β and IL-6 (Zhang *et al.*, 2011; Alt *et al.*, 2018). While our study examines macrophage inflammatory cytokine production mediated by hyperoxia and HMGB1, these studies examine the effects of GM-1111 on LL-37-induced inflammation in human keratinocytes and the nasal tissue of mice in a model of A. fumigatus induced rhinosinusitis (Zhang *et al.*, 2011; Alt *et al.*, 2018)

HMGB1 has been associated with increased TNF-α secretion suggesting a key role in mediating its release (Luan *et al.*, 2010; Kwak *et al.*, 2015; Cheng *et al.*, 2017, 2018) Thus, it is likely that GM-1111's suppression of cytokine secretion occurs through its neutralization of HMGB1.

While GM-1111 has been proven to bind to HMGB1 (data not shown) and a conformational change may be responsible for decreased binding to TLR4, it is possible that GM-1111 may also compete for the receptor. As we found that GM-1111 was able to inhibit downstream signaling stimulated by HMGB1 binding, we wanted to confirm its mechanism by determining if it was able to suppress NF-κB activation. As previously stated, TLR4 agonists such as HMGB1 and LPS bind to the receptor and activate NF-κB, causing increased transcription of proinflammatory cytokines. Previous studies have shown GM-1111 and similar compound GM-0111 to interrupt the binding of HMGB1 to TLR4 and RAGE (Zhang *et al.*, 2011; Savage *et al.*, 2016). These qualities were also demonstrated by heparin and ODSH (Rao *et al.*, 2010; Sharma *et al.*, 2014). Zhang et al found using a cell surface binding assay that GM-1111 was able to bind directly to RAGE (Zhang *et al.*, 2011). Our findings show that GM-1111 is able to suppress NF-κB activation at all concentrations (Fig.5). In this study, we demonstrate that GM-1111 reduces activation of NF-κB in cells exposed to both hyperoxia and LPS. Both LPS and hyperoxia were used to stimulate TLR4 activation and explore GM-1111's role in an infectious

model of VAP. Interestingly, GM-1111 significantly reduced NF-κB activation in LPS and hyperoxia stimulated macrophages despite not reducing extracellular HMGB1 suggesting that there is decreased binding of HMGB1 to TLR4. Our results are consistent with a previous study demonstrating a similarly structured molecule blocking TLR4 mediated signaling (Savage *et al.*, 2016). In that study, Raw 264.7 cells were stimulated with LPS and co-cultured with GM-0111. Treatment with GM-0111 at 500-10,000 μg/ml were able to significantly dampen IL-6 secretion (Savage *et al.*, 2016). NF-κB activation was also measured using Hek-Blue cells treated with LPS or Pam3CsK4 and GM-0111. In this study, 300-30,000 μg/ml of GM-0111 was able to reduce TLR4 activation while 0.1-10 μg/mL of GM-0111 was sufficient to suppress TLR2 activation (Savage *et al.*, 2016). This supports that GM-1111 and similarly structured compounds are able to inhibit TLR4 signaling and suggests that there may be greater specificity for TLR2 (Savage *et al.*, 2016). As GM-1111 has a higher specificity for TLR 2 than TLR4, that may be why NF-κB levels were not suppressed to the levels of hyperoxic controls. As previously mentioned, there may also be molecular ratio-based effectiveness GM-1111's ability to restore HMGB1-mediated phagocytic dysfunction which may apply to its effects on NF-κB activation (Figure 6). While GM-1111 was able to significantly dampen NF-κB activation, the levels do not return to hyperoxia control levels. Previous literature has shown LPS

stimulation to enhance acetylation of HMGB1 and its subsequent release from the cytoplasm (Wu *et al.*, 2012; CHEN *et al.*, 2013; Lu, Antoine, *et al.*, 2014; Yang *et al.*, 2014). In combination with hyperoxia, this may create an environment in which there is a high amount of HMGB1, where GM-1111 can reduce NF-κB activation but not fully suppress it by binding. While we have shown that GM-1111 can directly neutralize HMGB1's effects, our data suggests that extracellular HMGB1 levels do not play a significant role in NF-κB activation at least not at 24 hours of hyperoxia exposure or with this experimental design. These results correlate with findings by Wong and colleagues in which cultured human epithelial cells were exposed to hyperoxia whereby there was no effect on NF-κB activation (Wong *et al.*, 2002). Hyperoxia while causing significant increased extracellular HMGB1 accumulation, did not significantly activate NF-κB in our study, suggesting that LPS and its binding to PRRs is a critical factor to NF-κB activation which may be exacerbated by hyperoxic exposure. Thus, we propose that this suppression of NF-κB activity is not solely due to binding of HMGB1, but perhaps also due to competitive binding of TLR4. This inhibition of NF-κB activity and neutralization of HMGB1 is likely the mechanism by which TNF-α secretion is reduced. It also possible that extracellular HMGB1 alone or at least the amounts released by Raw-Blue cells in 24 hours are not sufficient enough to produce the phosphatase needed for the reaction

used in the Quantiblue assay. Perhaps use of recombinant HMGB1 or a longer hyperoxia treatment time is needed to demonstrate NF-κB activation solely from the extracellular HMGB1 accumulated during hyperoxic treatment. Assuming that the effect of GM-1111's neutralization of HMGB1 is a negligible factor, GM-1111's suppression of NF-κB activity seems to be strongly dependent on interrupting the binding between LPS or LPS/HMGB1 complexes to RAGE and TLR receptors.

Conclusion

In conclusion, GM-1111 may act via a dual mechanism to return function and inhibit downstream pro-inflammatory action by neutralizing HMGB1 and interrupting its receptor binding respectively. We propose that it may have potential applications in improving the host defense of immunocompromised individuals such as those with VAP and HMGB1-mediated conditions such as cystic fibrosis. Administration of this compound protects against HMGB1 compromised phagocytic dysfunction and this effect may be reproducible in other areas of the body. It may be most beneficial to administer treatment when the area is in the early stages of pathogenesis and exhibiting high levels of extracellular HMGB1. In non-injured lungs, administration of GM-1111 may slightly reduce phagocytic function due to potential binding to TLR4 receptors that are needed for phagocytosis. Due to this, structural modifications may need

to be made to allow for HMGB1 binding but reduce binding to TLR's. In other organ tissues with fewer resident immune cells, GM-1111's ability to bind to these receptors may not pose as issue. GM-1111 has been shown to reduce inflammation and protect the function of macrophages and may also have efficacy in reducing bacterial load due to potential antibacterial properties (Savage *et al.*, 2016). As this molecule is able to be nebulized, it may serve as a novel therapy to improve outcome for patients with VAP.

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