GAT107 IMPROVES SURVIVAL BY ATTENUATING HYPEROXIA-COMPROMISED HOST DEFENSE IN BACTERIAL CLEARANCE IN THE LUNGS VIA ALLEVIATING REDOX IMBALANCE IN MACROPHAGES

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ABSTRACT
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Alex G. Gauthier

Prolonged exposure to hyperoxia can compromise macrophage bacterial clearance functions, contributing to the increased susceptibility to pulmonary infections observed in hospital and ventilator-associated pneumonia (HAP/VAP). Previously studies in our lab demonstrate that activation of the α7 nicotinic acetylcholine receptor (α7nAChR) has protective effects against proinflammatory lung injury in animals subjected to prolonged hyperoxic exposure and bacterial infections. In this study, we demonstrated that administration of GAT107, an α7nAChR agonistic positive allosteric modulator (ago-PAM), improved survival by attenuating hyperoxia-compromised bacterial clearing functions of mice with Pseudomonas aeruginosa (PA) lung infection. GAT107 decreased the bacterial burden in mouse lungs by attenuating the hyperoxia-compromised phagocytic functions of both cultured macrophages, RAW 264.7 cells, and those isolated from mice. In hyperoxia-exposed RAW macrophages, GAT107 treatment attenuated oxidation of F-actin, a critical cytoskeletal component involved in phagocytosis. In hyperoxia-compromised mice with PA lung infection, GAT107 ameliorated hyperoxia-induced oxidative stress levels and increased the antioxidant oxidant potential in lung tissues and also in cultured macrophages. GAT107 induced the activation of the master antioxidant transcription factor, Nrf2, and significantly increased the levels of its down-stream antioxidant heme oxygenase-1 (HO-1) protein in macrophages. In macrophages,
hyperoxia induced mitochondrial membrane perturbation and also elevated mitochondrial superoxide (mitoSOX) levels, which was attenuated with GAT107 treatment. GAT107 decreased mitoSOX via attenuating hyperoxia-compromised manganese superoxide dismutase (MnSOD) activity and had no effect on its total protein levels. MnSOD activity was found to be strongly correlated with the extent of cysteine glutathionylation, which was attenuated in GAT107-treated macrophages. Thus, these data suggest that GAT107 provides protective effects against PA infection-induced inflammatory lung injury via attenuating hyperoxia-impaired macrophage function via restoring redox balance. GAT107-elicited efficacy in macrophage functions under hyperoxic conditions is through restoring the redox imbalance mediated partly by mitochondrial stress and MnSOD functions. Thus, GAT107 may be a potential therapeutic agent acting to improve host defense functions in the subjects exposed to prolonged periods of hyperoxia, such as VAP, HAP, and COVID-19.
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1. INTRODUCTION

1.1. Hyperoxia-Induced Compromise of Host Defense Functions and Ventilator-Associated Pneumonia

Oxygen therapy, using concentrations of supplemental oxygen up to 100% (hyperoxia), is a routine treatment for intensive care units (ICU) patients, surgical patients, pre-term neonates, patients with acute lung injury (ALI) / acute respiratory distress syndrome (ARDS), patients requiring home oxygen therapy care such as those with chronic obstructive pulmonary disorder (COPD), and in patients receiving supportive care for airway-associated infections, such as the novel COVID-19 coronavirus (Branson, 2018; Magnet et al., 2017; Renda et al., 2018; Walsh and Smallwood, 2017; Wang et al., 2020). Although oxygen therapy is a lifesaving intervention, prolonged exposure to hyperoxia to patients can compromise lung host defense and cause inflammatory acute lung injury (ALI) (Kallet and Matthay, 2013; Pham et al., 2017). Consequently, patients with compromised host defense and exposed to hyperoxia, experience a higher susceptibility to pulmonary bacterial infections that cause ventilator-associated pneumonia (VAP) and hospital-acquired pneumonia (HAP) (Bassi et al., 2014; Oliveira et al., 2014). Approximately one-third of all MV ICU patients develop VAP, which has a 4.6% mortality rate (Rello, 2005; Rello et al., 2002; Spalding et al., 2017). Oftentimes, Medicaid denies insurance claim reimbursement for these patients since the disease was acquired under hospital care, placing increased financial burden on both the patient and the hospital (Rello et al., 2002; Spalding et al., 2017). VAP patients have to pay large amounts of out-of-pockets expenses to cover the increased medical costs for care, which ranges from about
$20,000 to $100,000 and averages approximately $40,000 per case of VAP (Kollef et al., 2012; Rello et al., 2002; Zimlichman et al., 2013).

The antimicrobial functions of the innate immune defense system of the lung is complex and depends on the type of pathogen or pathogen/damage associated molecular pattern (P/DAMP) and their location within the airways (Martin and Frevert, 2005). Larger particle pathogens that enter the nasopharynx or conducting airways can become trapped in mucociliary surfaces where they can be expelled through a mucociliary clearance mechanism (Martin and Frevert, 2005). Smaller substances of 1µm diameter or less can be deposited into the deeper conducting airways of the bronchioles and alveoli. In the alveoli, the leukocyte population is composed of 95% macrophages (60-70% alveolar and 30-40% are interstitial macrophages), 1-4% lymphocytes, and 1% neutrophils (Martin and Frevert, 2005; Tan and Krasnow, 2016). Alveolar macrophages are a first line of defense against invading pathogens that enter the distal airways (Allard et al., 2018; Byrne et al., 2015; Craven et al., 1986). The alveolar environment is enriched with high levels of opsonizing immunoglobulins (e.g. IgG and IgA) and lipid binding protein, which augment the host defense response of alveolar macrophages to phagocytose invading pathogens (Martin and Frevert, 2005).

It is estimated that average adult intake of total air is approximately 10,000 L per day, which contains 2,100 L of oxygen (Hartl et al., 2018). In patients receiving oxygen therapy, oxygen concentrations often reach 100%, increasing the amount of inhaled oxygen to 10,000 L. Unlike other organs that rely on oxygenation via perfusion by circulating blood, the alveolar macrophages are in direct contact with these hyperoxic
levels of oxygen. Alveolar macrophages are derived from yolk-sac fetal monocytes, are self-renewing, and can be replenished by interstitial macrophage proliferation and differentiation (Tan and Krasnow, 2016). In response to the exposure to hyperoxia, the amounts of alveolar macrophage do not change due to the activation of cell survival pathways (Baleeiro et al., 2003; Monick et al., 2008; Petrache et al., 1999). However, in mice and ex vivo alveolar macrophage cultures, prolonged exposure to hyperoxia impairs the phagocytosis of bacteria known to produce VAP, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and certain bacteria from the *Enterobacteriaceae* family (Baleeiro et al., 2003; Barbier et al., 2013; Bassi et al., 2014; Chastre and Fagon, 2002).

*Pseudomonas aeruginosa* (PA)-induced pneumonia has the highest associated-death rates (87%) when compared to all other bacterial pneumonias (55%) (Chastre and Fagon, 2002; Cotoia et al., 2020). In VAP patients, PA also has the highest associated-death rates (65%) when compared to all other bacterial species (31%) (Chastre and Fagon, 2002; Cotoia et al., 2020). PA is a multi-drug resistant organism, which is normally innocuous in the environment, but can become highly opportunistic in immunocompromised patients (Bassetti et al., 2018). In ventilated patients receiving oxygen therapy, PA enters the lung usually by contact with contaminated surfaces on endotracheal tubes, drainage cuffs, or by direct contact of healthcare workers (Cotoia et al., 2020). Upon colonization of PA in the lung, the bacteria utilize a variety of virulence factors that cause the breakdown of lipids, elastic fibers, and proteins, activate quorum sensing, and elicit an inflammatory response (Hauser, 2011). As a consequence, PA pneumonia can disrupt or destroy the alveolar-capillary barrier,
resulting in pulmonary edema, hypoxemia, and respiratory failure (Chastre and Fagon, 2002; Cotoia et al., 2020; Hauser, 2011).

Currently, VAP patients with PA pneumonia are treated with cocktails of antibiotics including aminoglycosides, cephalosporin, carbapenem, and other broad-spectrum antibiotics (Bassetti et al., 2018; Cotoia et al., 2020). However, treatment with these antibiotic cocktails for PA pneumonia in VAP has yielded unconvincing clinical results of the efficacy of this therapeutic strategy (Cotoia et al., 2020). Thus, other therapies that can target more efficient clearance of these pathogenic organisms is critically needed. Since alveolar macrophages are a first line of defense in the airways against invading pathogens like PA, impairment of macrophage functions cause the increased the susceptibility to bacterial infections in animals and humans exposed to hyperoxia (Baleeiro et al., 2003; Patel et al., 2013; Reddy et al., 2009; Sitapara et al., 2014). Therefore, alveolar macrophages are a potential therapeutic target to improve outcomes for individuals that are immunocompromised by hyperoxic oxygen therapy.

1.2. Impact of Hyperoxia on Macrophage Function
The prolonged exposure of macrophages to hyperoxia induces the elevation of intracellular reactive oxygen species (ROS) (Freeman et al., 1982). ROS produce a phenomenon known as oxidative stress, a pathophysiological condition characterized by the relative higher amount of oxidants to reductants and their correlation with disease pathogenesis (Burton and Jauniaux, 2011). Free radicals derived from oxygen and other ROS can be controlled through the containment of antioxidant defense systems (Aerts et al., 1995; Birben et al., 2012; Cho et al., 2002; Gore et al., 2010). The
complex network of antioxidant molecules and enzymes in the cell are critical for mitigating oxidative stress and maintain cellular homeostasis (Birben et al., 2012). However, prolonged exposure to hyperoxia results in an overwhelming and continuous production of ROS, which can surmount the endogenous antioxidant defense system and induce mitochondrial damage (Freeman et al., 1982; O’Donovan and Fernandes, 2000; Pagano and Barazzone-Argiroffo, 2003). Mitochondrial damage by hyperoxic exposure is partly due to the generation of ROS, such as superoxide and hydroxyl radicals (Goscin and Fridovich, 1973; Guo et al., 2013; Indo et al., 2015; Majima et al., 1998; Morrow et al., 2007). Superoxide is generated by the one electron reduction of molecular oxygen and has a half-life of approximately 1-4 µs (Gielis et al., 2017). Superoxide is commonly generated as a reaction byproduct from cytochromes and enzymes containing iron-cores, such as the complexes of the electron transport chain (ETC) (Li et al., 2013). Upon the exposure to hyperoxia, intramitochondrial levels of oxygen increase (Düssmann et al., 2017), which may augment the basal mitochondrial superoxide byproduct formation. Importantly, hyperoxia exposure has been shown to decrease the activities of complex I, II, and IV of the ETC, which results in increased amounts of electron leakage, and the subsequent reduction of molecular oxygen to superoxide anion (Resseguie et al., 2015). Superoxide can then be converted to hydrogen peroxide in the mitochondria by manganese superoxide dismutase (MnSOD or SOD2) and by copper-zinc SOD (CuZnSOD or SOD1) in the cytosol (Wang et al., 2018). Hydrogen peroxide can then be either detoxified into water by catalase or permeate out of the mitochondria and contribute to cytosolic ROS and cause oxidative stress (Birben et al., 2012; Guo et al., 2013). Excessive
mitochondrial superoxide accumulation can cause oxidative damage to ETC enzymes and affects the levels and localization of electron carriers (e.g. NADH, FADH$_2$, cytochrome C) that are involved in oxidative phosphorylation, resulting in impaired energy production, changes in the mitochondrial membrane potential, and the additional production of ROS (Bhatti et al., 2017; Guo et al., 2013; Zorova et al., 2018). As a consequence, impaired energy production and excessive ROS has profound impacts on the phagocytic function of macrophages (Okpala et al., 2015; O’Reilly et al., 2003). Macrophage phagocytosis requires readily available pools of ATP (Borregaard and Herlin, 1982), which is depleted due to the lack of functional mitochondria under hyperoxic conditions (Monick et al., 2008).

In response to invading pulmonary pathogens, macrophages attempt to eradicate these microbes through the activation of bactericidal enzymes like NADPH oxidase (NOX2) (Griffith et al., 2009). NOX2 enzymes utilize oxygen as a substrate to generate superoxide anion in the cytosol and in the phagolysosomes of phagocytosed pathogens (Griffith et al., 2009; Parinandi et al., 2003). In pulmonary epithelial and endothelial cells, exposure to hyperoxia results in the increase of cytosolic superoxide levels, which was found to be dependent on NOX2 and NOX4 activation (Parinandi et al., 2003; Zhang et al., 2003). In hyperoxia-exposed NOX2 null mice, NOX4 activation contributes to alveolar capillary barrier dysfunction, pulmonary edema, and inflammatory lung injury (Griffith et al., 2009; Pandyala et al., 2009).

Thus, the combination of mitochondrial generated ROS that permeates into the cytosol and also ROS generated in the cytosol contribute to total increase in the hyperoxia-induced elevated cytosolic ROS levels in macrophages. As a consequence,
high cytosolic ROS levels can cause the oxidation of proteins and lipids resulting in the post-translational modification (PTM) of F-actin fibers (Morrow et al., 2007; O’Reilly et al., 2003). F-actin is a critical cytoskeletal component that is used by macrophages to migrate to and form phagosomes around opsonized pathogens like PA (Hartwig and Yin, 1988). As a result, hyperoxia-induced F-actin oxidation contributes to the formation of stress fiber filaments, the dysregulation of actin polymerization, and impairs the migratory and phagocytosis functions of macrophages (Morrow et al., 2007; O’Reilly et al., 2003). The impaired phagocytic functions of hyperoxia-induced oxidative stress in macrophages can be attenuated by treatment with activators of antioxidant defense pathways like sulforaphane or with supplemental antioxidants such as ascorbic acid, n-acetyl cysteine, and exogenous superoxide dismutase (SOD) (Morrow et al., 2007; Patel et al., 2020). Importantly, antioxidant treatment protects against oxidation of F-actin filaments and restores the antibacterial and phagocytic functions of macrophages (Arita et al., 2007; Morrow et al., 2007; O’Reilly et al., 2003). Thus, the prolonged exposure to hyperoxia has profound deleterious effects on macrophage functions to migrate, phagocytose, and kill invading pathogens, which can be protected against by treatment with supplemental and activators of antioxidant pathways.

1.3. The Vagus Nerve Mediated Cholinergic Anti-Inflammatory Pathway
Alveolar macrophages perform their host defense functions, which are mediated in part by both autocrine and paracrine-like signaling of cytokines and chemokines. Indeed, the balance between pro- and anti-inflammatory cytokine and chemokine production has been previously identified as a neuromodulator mechanism known as
the cholinergic anti-inflammatory pathway (Borovikova et al., 2000). The cholinergic anti-inflammatory pathway is mediated by the vagus nerve, which is the 10th cranial nerve (Tracey, 2002). In response to damage, pathogens, or inflammatory signals, the afferent vagus nerve conduct signals to the central nervous system where they are integrated and relayed to effector organs via the efferent vagus nerve. Acetylcholine is released from the efferent vagus nerve innervating pulmonary neuroendocrine cells and can bind to macrophage α7 nicotinic acetylcholine receptors (α7nAChRs) and downregulate hyper-inflammatory responses, such as those during VAP (Pavlov et al., 2003). In addition, cholinergic anti-inflammatory pathway-mediated signaling has been identified as a missing link that plays a role in controlling autophagy and regulating cellular metabolic homeostasis (Chang et al., 2019; Lu et al., 2014).

1.4. α7nAChR as a Pharmacological Target for Attenuating Hyperoxia-Compromised Macrophage Functions

Previously, we and others have shown that the hyperoxia-compromised innate immune functions of alveolar macrophages are significantly attenuated by pharmacological activators of the α7 nicotinic acetylcholine receptors (α7nAChRs) (Ulloa, 2005; Wang et al., 2004). The α7nAChR is a homomeric pentamer and a ligand-gated ion channel found on both neuronal and non-neuronal cells (Báez-Pagán et al., 2015; Bagdas et al., 2016; Papke et al., 2018). Upon stimulation with endogenous acetylcholine, choline or other agonists, there is a rapid influx of calcium ions into neurons that activate certain calcium-dependent pathways (Báez-Pagán et al., 2015; Hoover, 2017). Evidence suggests that the activation of non-neuronal cells, such as macrophages, produces an influx of calcium ions but does not significantly change whole cell currents (Skok, 2009; Villiger et al., 2002). A number of studies
have shown that the activation of peripheral α7nAChR produces anti-inflammatory effects (de Jonge et al., 2005; Papke et al., 2018; Wang et al., 2003). It has been postulated that the anti-inflammatory effect produced by the activation of α7nAChR on peripheral immune cells such as macrophages may be due to: 1) inhibition of the phosphorylation of the transcription factor STAT3, which subsequently decreases the activation of certain genes that produce inflammatory cytokines (Báez-Pagán et al., 2015); 2) activation of the PI3K/Akt/Nrf2 antioxidant pathway and induction of heme oxygenase-1 (HO-1) and 3) the inhibition of NF-κB p65 subunit phosphorylation and subsequent nuclear-translocation through STAT3-NF-κB convergence (Báez-Pagán et al., 2015; Hoover, 2017).

Previously, our lab has shown that administration of GTS-21, a partial agonist of the α7nAChR, significantly increased bacterial clearance and decreased lung injury in hyperoxia-exposed mice with PA pneumonia (Sitapara et al., 2014). Importantly, GTS-21 activates the cholinergic anti-inflammatory pathway, which in turn has been shown to attenuate the phagocytic function of macrophages compromised by the prolonged exposure to hyperoxia (Sitapara et al., 2014). However, other clinically relevant endpoints, such as mortality rates, were not significantly affected by GTS-21 administration (unpublished results). Moreover, treatment with α7nAChR agonists like acetylcholine, attenuates mitochondrial perturbation in LPS-primed mouse primary macrophages (Lu et al., 2014). Yet, it remains unclear if macrophage innate immune functions can be improved by attenuating mitochondrial dysfunction. Consequently, we wanted to ascertain the efficacy of a next generation α7nAChR
activator in animals and in an *in vitro* model, where macrophage functions are compromised by hyperoxia exposure.

### 1.5. GAT107 and Agonistic Positive-Allosteric Modulators

In this study, we determined the efficacy of the (+) - enantiomer of racemic 4-({4-bromophenyl}-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline-8-sulfonamide, GAT107, to attenuate hyperoxia-induced impairment of host innate immune functions and its underlying mechanisms. GAT107 is positive allosteric modulator (PAM) and direct allosteric activator (DAA) that 1) augments or potentiates the response to orthosteric site ligands and 2) activates the α7nAChR ion channel (in the absence of an orthosteric agonist) by binding to an allosteric site distinct from that of the PAM site (Bagdas et al., 2016; Horenstein et al., 2016; Papke et al., 2018, 2014; Thakur et al., 2013). *In vitro*, the α7nAChR can be rapidly desensitized by acetylcholine (ACh). Interestingly, the coadministration of GAT107 with ACh produces a significant decrease in the ACh-induced desensitization (Horenstein et al., 2016, 2016; Papke et al., 2014). It is likely that GAT107 facilitates the conversion of desensitized states to conducting states which surmounts receptor desensitization (Horenstein et al., 2016, 2016; Papke et al., 2014).
1.6. Hypothesis

GAT107 improves the survival of hyperoxia-compromised mice challenged with *Pseudomonas aeruginosa* (PA) lung infection via enhancing host defense functions of macrophages.

Aim 1: To determine if GAT107 improves survival in mice exposed to hyperoxia and challenged with PA lung infection.

Aim 2: To determine if GAT107 attenuates hyperoxia-compromised host defense of PA bacterial clearance in the lungs of mice.

Aim 3: To determine if GAT107 attenuates hyperoxia-induced phagocytic dysfunction modulating redox imbalance in macrophages.

Aim 4: To determine if GAT107 can ameliorate hyperoxia-induced mitochondrial redox imbalance and dysfunction.
2. MATERIALS AND METHODS

2.1. Cell Culture and Special Reagents
Murine macrophage–like RAW 264.7 cells (TIB-71; American Type Culture Collection (ATCC), Manassas, VA) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) and supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA). Cells were maintained at 37°C in normoxia (5% CO₂/21% O₂) for 24 h, allowed to grow to 70–80% confluency and subcultured every 2 days. Bone marrow was harvested from 6-8-week-old male C57BL/6 mice (Jackson laboratories), isolated, and cultured to allow for differentiation into bone marrow-derived macrophages (BMDM), as previously described (Weischenfeldt and Porse, 2008). Hyperoxic exposure was performed in sealed, humidified chambers (Billups-Rothenberg Inc., Del Mar, CA) flushed with 95% O₂/5% CO₂ at 37°C. An oxygen analyzer (MSA Medical Products, Pittsburgh, PA) was used to monitor the O₂ levels.

2.2. Animal Studies
Male C57BL/6 mice (6 to 10 weeks old; The Jackson Laboratory, Bar Harbor, ME, USA) were used in this investigation based on a protocol (protocol #1953) approved by the Institutional Animal Care and Use Committees of St. John’s University. The mice were housed in a specific pathogen-free environment, maintained at 22°C in ≈50% relative humidity and with a 12 h light/dark cycle. All mice had ad libitum access to standard rodent food and water. Mice were randomized to receive either 3.3 mg/kg of ((+)-4-(4-bromophenyl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline-8-sulfonamide, GAT107, donated by Dr. Ganesh S. Thakur, Northeastern University, Boston, MA, or saline, administered by intraperitoneal injection at 24, 36, and 48 h
after the onset of hyperoxic exposure. After 48 h of exposure, the mice were inoculated with $0.1 \times 10^8$ colony-forming units (CFUs) of *Pseudomonas aeruginosa* (PA) by making a 1- to 2-cm incision on the neck to expose the trachea after anesthetization with sodium pentobarbital (75 mg/kg). PA was used as the selected pathogen as it is associated with 21% of all VAP cases (Richards et al., 1999). Twenty-four hours after bacterial inoculation, mice were euthanized via exsanguination and bronchoalveolar lavage (BAL) and lung tissues were immediately collected as described previously (Patel et al., 2013). For the collection of BAL, the lungs were lavaged with PBS, and afterwards, the lungs were excised and immediately placed into 1 mL cold PBS containing a protease and phosphatase inhibitors cocktail (Pierce Thermo Scientific) followed by homogenization by a Dounce tissue homogenizer. For animal survival studies, mice were subjected for 72 h of hyperoxia exposure and inoculated similarly as stated above. Mice were then monitored every hour over the course of 24 h for survival.

### 2.3. Exposure to Hyperoxia

Male C57BL/6 mice and cultured macrophages were exposed to hyperoxia as previously described (Patel et al., 2013). Briefly, animals were placed in microisolator cages (Allentown Caging Equipment, Allen-town, NJ, USA), which were kept in a Plexiglas chamber (BioSpherix, Lacona, NY, USA) and exposed to ≥95% O$_2$ for up to 48 h. The exposure of murine macrophage RAW 264.7 cells was conducted in humidified Plexiglas chambers (Billups-Rothenberg, Del Mar, CA, USA), flushed with 95% O$_2$/5% CO$_2$ at 37°C for 24 h. An oxygen analyzer (MSA; Ohio Medical
Corporation, Gurnee, IL, USA) was used to monitor the O$_2$ concentration in the chamber.

2.4. Bronchoalveolar Lavage and Protein Quantification

Murine bronchoalveolar lavage (BAL) fluid was obtained as described previously (Patel et al., 2013). Briefly, mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (75 mg/kg). After a 1- to 2-cm incision was made on the neck, the trachea was dissected, and a 20-gauge × 1.25-inch intravenous catheter was inserted caudally into the lumen of the exposed trachea. The lungs were gently lavaged twice with 1 mL sterile, nonpyrogenic phosphate-buffered saline (PBS) solution (Mediatech, Herndon, VA, USA) containing a cocktail of protease and phosphatase inhibitors (Thermo Pierce Scientific). BAL samples were centrifuged at 200 x g at 4°C for 5 minutes, and the resultant supernatants were stored flash frozen in liquid nitrogen and then stored in a freezer at −80°C. Total protein content in BAL was determined by using the Pierce Bicinchoninic acid (BCA) assay Kit (Thermo Fisher, Waltham, MA) as per the manufacturer’s instructions.

2.5. Quantitative Bacteriology

Lung homogenate and BAL were serially diluted, and the viable bacterial counts were determined using a colony formation unit (CFU) assay by plating onto Pseudomonas Isolation Agar (Difco, Sparks, MD), and incubating at 37°C for 18 h.

2.6. Assay for Oxidative Stress and Antioxidant Potential

Oxidative stress was determined by measuring the oxidation–reduction potential (ORP) using the RedoxSYS Diagnostic System (Luoxis Diagnostics, Inc., Englewood, CO). Lung homogenate was evaluated for its oxidative-reduction potential (ORP)
reported in millivolts (mV) and the capacity of the ORP (cORP) also known as the antioxidant potential measured in µcoloums (µC) at room temperature, using the protocol provided by the manufacturer.

2.7. Measurement of SOD1 Activity

RAW 264.7 cells were seeded in 6-well plates, exposed to hyperoxia, and treated as with GAT107 (3.3 µM) for 24h. After 24h, cells were washed 3x in PBS and lysed with cell lysis buffer (Cell Signaling Technology, Danvers, MA) that contained Halt protease and phosphatase inhibitor cocktail (Thermo Fischer, Waltham, MA). The total protein content of cell lysate was determined by using the Pierce Bicinchoninic acid (BCA) assay Kit (Thermo Fisher, Waltham, MA) as per the manufacturer instructions. Equal amounts of total protein from non-denatured samples were loaded onto 12% native-PAGE and separated at 100V at 4˚C for 90 minutes. Native-PAGE gels were then washed with 3x distilled water and then incubated with 2.43 mM nitrotetrazolium blue (Acros Chemical), 2.85 µM riboflavin (Sigma), and 28 mM TEMED (GE Healthcare) for 20 minutes, protected from light, and at room temperature, as described (Beauchamp and Fridovich, 1971). After 20 minutes, gels were placed on a light box and allowed to develop. The presence of achromatic bands represents the inhibition of the reduction of nitro tetrazolium blue by the SOD1 enzymatic and was detected by Bio-Rad ChemiDoc.

2.8. 2’,7’-Dichlorofluorescin diacetate (DCFH-DA) reactive oxygen species (ROS) detection assay

RAW 264.7 cells were seeded in quadruplicate in 96-well black wall, clear bottom plates, exposed to hyperoxia, and treated as previously described. After 24 h, cells
were analyzed using the DCFH-DA assay (Cell Biolabs, San Diego, CA), according to manufacturer instructions. In brief, cells were incubated with 1 mM DCFH-DA for 30 minutes at 37°C, then washed three times with warm PBS. The presence of total intracellular DCF (an indicator for ROS levels) was determined by lysing cells, as described previously (Patel 2020), and measuring a green fluorescent signal at 480/530nm, using a Biotek Synergy LX multimode reader (Winooski, Vermont, USA). DCF relative fluorescent units were then reported as a percent relative to the 21% O₂ exposure group (normoxic or control group).

2.9. Phagocytosis Assay and Assessment of Actin Stress Filament Formation

The phagocytosis assay was performed as previously described, with minor modifications (Morrow et al., 2007; Sitapara et al., 2014). Briefly, RAW 264.7 cells or BMDMs were seeded in 24-well plates and allowed to adhere for 6 h followed by exposure to 95% O₂ in the absence or presence of GAT107 (3.3 µM) for 24 h. RAW 264.7 cells were incubated at 37°C for 1 h with opsonized FITC-labeled latex beads (Polysciences, Warrington, PA) at a ratio of 100 : 1 (beads : cell). Macrophages were incubated with 0.04% Trypan blue in PBS for 10 minutes to quench the beads that were not internalized by the macrophages. To visualize the uptake of FITC-labeled latex beads, macrophages were fixed with 4% paraformaldehyde for 10 minutes, washed with PBS, and stained with 4′,6-diamidino-2-phenylindole (DAPI, Molecular Probes, Eugene, OR). To visualize the cell cytoskeleton, cells were stained with rhodamine phalladoin (Molecular Probes, Eugene, OR). The phagocytosis or uptake of the beads was assessed visually using an Evos Fluorescent Microscope (Thermo
Fischer, Waltham, MA) followed by counting 200 individual macrophages per well in duplicates from three independent experiments by a blinded person for each experimental group. To assess the hyperoxia-induced modification of actin polymerization, a modified experiment method was used (O’Reilly et al., 2003). Using the fluorescent micrographs obtained from the above phagocytosis assay, a Fiji ImageJ analysis (version 2.0) with the JACoP plugin was used to determine a Mander’s Correlation Coefficient using thresholds for the amount of phalladoin signal associated with the DAPI signal. This value was converted to percentage to estimate the amount of polymerized F-actin and stress filament formation.

2.10. Protein Carbonyl Formation

The amount of total protein oxidation and carbonyl formation was determined by using a commercially available Oxidized Protein Western Blot Kit (Abcam, Cambridge, UK) based on the manufacturer instructions. Equal amounts of whole cell lysate from three to four independent experiments were subjected to a protein carbonyl derivatization with 2,4-dinitrophenylhydrazone (DNP-hydrazone) in the presence of DNP-hydrazone or left unreacted as a negative control. The samples were separated by 12% SDS-PAGE and transferred to PVDF membranes. The membranes were washed and blocked in 5% milk/TBST solution. Next, membranes were washed and incubated with secondary anti-DNP antibody, washed, and the immunoreactive DNP bands were detected using enhanced chemiluminescence (ECL) reagents (Pierce Thermo Scientific) by a Bio-Rad ChemiDoc XRS+ system (Biorad, Hercules, CA).
2.11. Western Blot Analysis

For intracellular protein analysis, cells were washed 3x in PBS and lysed with cell lysis buffer (Cell Signaling Technology, Danvers, MA) that contained Halt protease and phosphatase inhibitor cocktail (Thermo Fischer, Waltham, MA). The total protein content of cell lysate was determined by using the Pierce Bicinchoninic acid (BCA) assay Kit (Thermo Fisher, Waltham, MA) as per the manufacturer instructions. Samples were loaded onto 12% or 15% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA) and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Nonspecific binding sites on the membrane were blocked by incubating the membrane with either 5% nonfat dry milk (Bio-Rad, Hercules, CA) in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST) for 1h at room temperature. Subsequently, membranes were washed three times with TBST and incubated overnight at 4°C with anti-HO-1 (1:1000, #ab13248, Abcam, Cambridge, UK), anti-MnSOD (1:1000, #AD-SOD-110, Enzo, New York, NY), and anti-pan-actin (1:1000, #8456, Cell Signaling) antibodies diluted in 5% nonfat dry milk in TBST. After three washes with TBST, the membranes were incubated with goat anti-rabbit horseradish peroxidase-coupled secondary antibody (1:5,000; GE Healthcare, Chicago, IL) for 1 h at room temperature. Subsequently, membranes were washed three times again with TBST and the immunoreactive proteins were visualized using the SuperSignal West Pico Plus Chemiluminescent Substrate (Thermo Fisher, Waltham, MA) per the manufacturer instructions. The images were developed using Bio-Rad ChemiDoc XRS (Bio-Rad, Hercules, CA). Immunoreactive bands were quantified using ImageJ software (version 2.0.0).
2.12. Nrf2 Analysis

Nrf2 activation was determined by determining the amount of immunofluorescent Nrf2 nuclear colocalization. RAW 264.7 cells were seeded and treated as described in the DCFH-DA experiment. After 24h, cells were permeabilized 0.1% Triton X-100 (Millipore Sigma, St. Louis, MO) made in PBS, blocked 10% goat serum (Millipore Sigma) in PBS, and incubated with anti-Nrf2-antibody (donated by Dr. Ed Schmidt of Montana State University) overnight. Cells were washed and immunoreacted with AlexaFluor488 (Pierce Thermo Scientific). Fluorescent micrographs were captured using an Evos fluorescent microscope. The images were analyzed for the amount of Nrf2 localized in the nucleus using a Mander’s Correlation Coefficient with thresholds as described above in the actin stress filament formation (see Section 2.9). The amount of Nrf2 signal located within the same signal as the nucleus was used as a marker of Nrf2 activation. The Mander’s correlation coefficient were then reported as a percent of total Nrf2 signal within the nucleus.

2.14. Measurement of Mitochondrial Membrane Potential and Mitochondrial Superoxide

RAW 264.7 cells were seeded in 96-well black wall, clear bottom plates, exposed to hyperoxia, and incubated with and without GAT107 (3.3 µM) as previously described. Macrophage mitochondrial membrane potential was determined by using the tetramethylrhodamine ethyl ester (TMRE) Mitochondrial Membrane Potential Assay Kit (Abcam, Cambridge, UK) as per the manufacturer’s instructions. In brief, after the 24 h incubation period, cells were incubated with 1 µM of TMRE for 20 minutes at 37°C. Prior to TMRE, a negative control group for mitochondrial membrane
polarization was generated by incubating the cells with 20 µM of the carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) uncoupling reagent for 15 minutes at 37°C. After incubation with TMRE, cells were gently washed with PBS containing 0.2% BSA and fluorescence detected at 549nm/570nm using a Synergy LX multimode reader. In another set of experiments, macrophages were cultured and treated as described for the TMRE assay and then incubated with MitoTracker Green FM (Thermo Fisher, Waltham, MA) to determine the mitochondrial density as per the manufacturer’s instructions. MitoTracker Green FM accumulates in mitochondria, independent of the membrane potential, and serves as a marker for total mitochondrial density. The relative fluorescent levels of MitoTracker Green FM in each condition group were then normalized to the 21% O₂ control group, and these values were used to normalize the percent TMRE relative fluorescence values, which was represented as a bar graph as the percent TMRE / MitoTracker Green to demonstrate the mitochondrial membrane polarization status. For the measurement of mitochondrial superoxide levels, after 24h of incubation, macrophages were incubated with 5 µM of the MitoSOX reagent (Invitrogen, Carlsbad, CA) for 10 minutes at 37°C and washed three times with 37°C PBS as per the manufacturer’s instructions. The presence of mitochondrial superoxide was detected using a Synergy LX multimode reader at the settings of 510 / 580 nm.

2.15. Measurement of MnSOD Activity

After 24 h of hyperoxic exposure and incubation with GAT107 (3.3µM), RAW 264.7 macrophage cell lysates were prepared as described above (Section 2.11). Equal
amounts of protein were tested for the enzymatic activity of MnSOD using a Superoxide Dismutase (SOD) Colormetric Activity Kit (Thermo Fisher) as per manufacturer’s instructions. In brief, non-mitochondrial iron SOD (FeSOD) and copper zinc SOD (CuZnSOD) were inhibited by incubating the cell lysate with cyanide. Cell lysates containing cyanide were then incubated with a xanthine oxidase reagent that generates superoxide in the presence of oxygen. The amount of superoxide not dismutated into hydrogen peroxide by MnSOD is then reacted with a nitro blue tetrazolium blue reagent to yield a yellow color. This chromogenic reaction product was read at 450 nm on a Synergy LX multimode reader (Biotek, Winooski, VT). The results were compared to a standard curve generated by using bovine erythrocyte SOD (produced without the addition of cyanide) and normalized to total protein concentrations.

2.16. Immunoprecipitation and Immunodetection

RAW 264.7 cells were exposed to 24h of hyperoxia and incubated with GAT107 (0 or 3.3 µM) as described above. Cell lysates were collected, and protein content determined as described for the Western blot analysis (section 2.11). MnSOD was co-immunoprecipitated from cell lysates using the Pierce Direct Magnetic IP/Co-Immunoprecipitation Kit (Thermo Scientific, Waltham, MA) using a polyclonal anti-MnSOD antibody (Enzo, New York, NY) per the manufacturer’s instructions. After immunoprecipitation, samples were immediately subjected to SDS-PAGE and Western blot analysis as described above (section 2.11). Western blot analysis was
conducted as described above using anti-MnSOD antibody (1:1000, Enzo, New York, NY) and monoclonal anti-glutathione antibody (1:1000, Virogen, Watertown, MA).

2.17. Statistical Analysis
The statistical analyses were carried out using GraphPad Prism statistical software (version 7.0a). The results are presented as the mean ± SEM. The data were analyzed for statistical significance according to analysis of variance (ANOVA) with Dunnett’s post hoc analysis. Animal survival data was analyzed by using a Kaplan-Meier curve. Glutathionylation versus MnSOD activity data was analyzed using a correlation analysis using a two-tail Pearson Correlation Coefficient test. A 95% confidence interval was used and a p-value less than 0.05 was considered significant.
3. FIGURES AND FIGURE LEGENDS

Figure 1. Systemic administration of GAT107 increases survival in mice with exposed to hyperoxia and challenged with PA lung infection

Male C57BL/6 were exposed to >95% O₂ for 72 h and then inoculated with PA (0.1 x 10⁸ CFUs / mouse) and then returned to 21% O₂ after inoculation. Mice were randomized to receive GAT107 (0 or 3.3 mg/kg) administered via intraperitoneal injection every 12 h starting at hour 24 of hyperoxia. After mice were inoculated, animal survival was monitored and recorded every hour for 24 h and represented above as a Kaplan-Meier curve. Data were analyzed using the log-rank Mantel-Cox test; *p < 0.05 compared to hyperoxia exposed vehicle treated control group (n = 5 - 10 mice / group).
Figure 2. Systemic administration of GAT107 improves bacterial clearance in mice exposed to 48 h hyperoxia and challenged with PA infection.

Male C57BL/6 were exposed to >95% O₂ (hyperoxia) for 48 h and then inoculated with PA (0.1 x 10⁸ CFUs / mouse) and then returned to 21% O₂ after inoculation. Mice were randomized to receive GAT107 (0 or 3.3 mg/kg) administered via intraperitoneal injection every 12 h starting at hour 24 of hyperoxia. BAL and lung tissue were harvested 24 h after inoculation. Viable bacteria in the airways and lungs were quantified by plating serials dilutions of (A) BAL and (B) lung homogenate and expressed as the log of colony forming units (CFUs) per mL. Data represent the mean ± SEM from n = 6-7 mice per group. Statistical differences were determined between all groups and indicated a **** p < 0.0001 compared to the hyperoxia exposed vehicle control group.
Figure 3. GAT107 restores hyperoxia-compromised macrophage phagocytic function in a macrophage cell line and in primary macrophages.

RAW 264.7 were either exposed to 21% O₂ (white bar) or 95% O₂ (hyperoxia) (black bar) with or without GAT107 (grey bars). Cells were incubated with FITC-labeled minibeads for 1 h and stained to visualize the cytoskeleton and nucleus. Immunofluorescent micrographs show the phagocytosed beads (green), cytoskeleton (red), and nucleus (blue) of (A) RAW 264.7 cells and (B) BMDMs. The bar graphs represent the quantification of phagocytosis of at least 200 cells was represented as a percent beads phagocytosed. Quantification of phagocytosis for (C) RAW 264.7 and (D) BMDMs cells. Each value represents the mean ± SEM of three independent experiments for each group. Statistical differences were determined between all groups and indicated a **** p < 0.0001, *** p < 0.001 compared to the hyperoxia exposed vehicle control group.
Figure 4. GAT107 Mitigates Hyperoxia-Induced Actin Oxidation and Alterations in Polymerization.

RAW 264.7 were either exposed to 21% O\(_2\) or 95% O\(_2\) (hyperoxia) with or without GAT107. After 24 h, (A) the actin polymerization was analyzed using fluorescent micrographs obtained as described in the Phagocytosis Assay in the methods, and the ratio of actin to the nuclei were reported as a percent of non-overlapping signals obtained from ImageJ’s co-localization analysis plugin and computation of the Manders’ Correlation Coefficient. Each value represents mean ± SEM of three independent experiments for each group. Statistical differences were determined between all groups and indicated a **** p < 0.0001, ** p < 0.01 compared to the hyperoxia exposed vehicle control group. (B) Macrophages were then also analyzed for total protein carbonylation by oxidized protein Western blot and the 43kD immunoreactive band was used to evaluate the extent of actin oxidation (as indicated by the arrow) for 3 – 4 independent experiments per group.
Figure 5. Systemic administration of GAT107 attenuates oxidative stress via enhancement of total lung antioxidant potential.

Male C57BL/6 mice were exposed to 95% O₂ (hyperoxia) for 48 h and then inoculated with PA (0.1 x 10⁸ CFUs / mouse), and then returned to 21% O₂ after inoculation. Mice were randomized to receive GAT107 (0 or 3.3 mg/kg) administered via intraperitoneal injection every 12 h starting at hour 24 of hyperoxia. Lung tissue was harvested 24 h after inoculation. Lung homogenate was analyzed for (A) oxidative-reduction potential (ORP) measured in millivolts (mV) and (B) capacity of the ORP or total antioxidant potential (cORP) measured in µCoulombs (µC) by the RedoxSys System. Data represent the mean ± SEM of two-independent experiments from n = 4 mice per group. Statistical differences were determined between all groups and indicated a *p < 0.05 compared to the hyperoxia exposed vehicle control group.
Figure 6. GAT107 Attenuates Hyperoxia-Induced Oxidative Stress by Restoring Hyperoxia-Compromised SOD1 Function.

RAW 264.7 were either exposed to 21% O₂ (room air) (white bar) or 95% O₂ (hyperoxia) (black bar) with or without GAT107 (grey bars). After 24 h, (A) macrophages were subjected to DCFH-DA Assay and the amount of fluorescent DCF signal within macrophages was determined spectrophotometrically and reported as a percent relative to the room air control to evaluate oxidative stress. (B) SOD1 activity was determined by gel-based nitro tetrazolium blue gel assay as described in the methods section and (C) reported as a fold change compared to the room air control group. Each value represents mean ± SEM of three independent experiments for each group. Statistical differences were determined between all groups and indicated a **** p < 0.0001, *** p < 0.001, ** p < 0.01 compared to the hyperoxia exposed vehicle control group.
Figure 7. GAT107 Activates the Nrf2/HO-1 Antioxidant Pathway.

RAW 264.7 were either exposed to 21% O₂ (white bar) or 95% O₂ (hyperoxia) (black bar) with or without GAT107 (grey bars). After 24h, macrophages were fixed, permeabilized, blocked, and incubated with a polyclonal anti-Nrf2-antibody overnight, then conjugated to AlexaFluor 488, and then counterstained with DAPI to visualize the nucleus. (A) Immunofluorescent micrographs were subjected to ImageJ co-localization analysis and pseudo colored to determine the amount of Nrf2 signal (red) localized to the nucleus (green) using the Manders’ Correlation Coefficient. (B) The bar graphs represent the percent amount of total Nrf2 signal localized to the nuclear region. Under the same experimental conditions, macrophages were also analyzed for levels of heme oxygenase-1 (HO-1) and actin in whole cell lysate by Western blot analysis. (C) The representative immunoreactive bands for HO-1 and actin and (D) the quantification of immunoreactive
bands normalized to actin. Each value represents the mean ± SEM of two to three independent experiments for each group. Statistical differences were determined between all groups and indicated a **p < 0.0001, * p < 0.05 compared to the hyperoxia exposed vehicle control group.
Figure 8. GAT107 restores hyperoxia-compromised macrophage mitochondrial integrity and mitochondrial oxidative stress.

RAW 264.7 were either exposed to 21% O$_2$ (white bar) or 95% O$_2$ (hyperoxia) (black bar) with or without GAT107 (grey bars). After 24 h, (A) macrophage mitochondrial integrity was measured by tetramethylrhodamine, ethyl ester (TMRE) mitochondrial membrane potential assay and normalized to MitoTracker Green. TMRE accumulates in mitochondrial directly proportional to the mitochondrial membrane potential, while MitoTracker Green localizes to mitochondria based on mitochondrial mass, not the polarization status. (B) Macrophages were then analyzed for mitochondrial superoxide using the MitoSOX dye after hyperoxia exposure. Each value represents mean ± SEM of three independent experiments for each group. Statistical differences were determined between all groups and indicated a **** p < 0.0001, ** p < 0.01 compared to the hyperoxia exposed vehicle treated control group.
Figure 9. GAT107 attenuates hyperoxia-compromised MnSOD activity.

RAW 264.7 were either exposed to 21% O₂ (room air) (white bar) or 95% O₂ (hyperoxia) (black bar) with or without GAT107 (grey bars). After 24 h, whole cell lysates were analyzed for total MnSOD levels by Western blot for MnSOD. (A) Representative Western blots for MnSOD and actin and (B) the densiometric analysis of Western blot bands. (C) RAW 264.7 cell lysate was used to determine MnSOD activity using a SOD activity kit in the presence of cyanide to inhibit non-MnSOD activities. Each value represents mean ±
SEM of three independent experiments for each group. \#p < 0.05, versus the room air control group; Statistical differences were determined between all groups and indicated a **** p < 0.0001, *** p < 0.001 compared to the hyperoxia exposed vehicle treated control group.
Figure 10. The hyperoxia-induced glutathionylation of MnSOD is significantly decreased by GAT107.

RAW 264.7 were either exposed to 21% O₂ (white bar) or 95% O₂ (hyperoxia) (black bar) with or without GAT107 (grey bars). After 24 h, whole cell lysates were co-immunoprecipitated for MnSOD. (A) Representative Western blots for immunoprecipitated MnSOD and conjugated GSH. (B) Densiometric analysis for immunoreactive GSH and MnSOD bands were determined and the ratio of GSH to MnSOD was reported as a percentage on the bar graph. Each value represents the mean ± SEM of 3-4 independent experiments for each group. Statistical differences were determined between all groups and indicated a **** p < 0.0001, ** p < 0.01 compared to hyperoxia exposed vehicle control group. (C) MnSOD activity was then compared to their samples
respective corresponding percentage of MnSOD glutathionylation and presented as a correlation plot. Each value on the correlation plot represents individual sample data points from 3–4 independent experiments. The measure of strength between the X and Y variables was determined by a two-tailed Pearson-correlation coefficient, which computed a $p < 0.0001$ between MnSOD activity and percentage of MnSOD glutathionylation.
Figure 11. Proposed pathway of the efficacy of GAT107 on macrophages to alleviate the bacterial burden in hyperoxia-compromised mice with PA lung infection.

The prolonged exposure to hyperoxia (O₂) induces the accumulation of reactive oxygen species (ROS) in the cytosol and mitochondria of macrophages. Macrophage mitochondrial normally generate superoxide (O₂⁻) as a byproduct of the electron transport chain, which is rapidly dismutated by manganese superoxide dismutase (MnSOD) into hydrogen peroxide (H₂O₂). However, under hyperoxic conditions, high concentrations of oxygen contribute to both mitochondria dysfunction and mitochondrial superoxide accumulation. Sustained high levels of superoxide compromise MnSOD activity via promoting cysteine glutathionylation, which contributes to increasing the total level of mitochondrial superoxide. In addition, cytosolic...
antioxidant defense systems (e.g. SOD1) are also suppressed during the prolonged exposure to hyperoxia. High levels of ROS accumulate in the cytosol, which oxidize F-actin filaments, resulting in impaired macrophage phagocytic functions. Together, both mitochondrial dysfunction and F-actin oxidation contribute to the impaired bacterial clearance functions of macrophages, resulting in the increased bacterial burden in the airways of mice with PA lung infection and higher mortality rates. Treatment with GAT107 to macrophages activates α7nAChR and results in the activation of the Nrf2. GAT107-induced Nrf2 activation upregulates heme oxygenase-1 (HO-1). Upregulated levels of HO-1 then contribute to alleviating the oxidative burden in the mitochondria and the cytosol. GAT107 then mediates the reduction in mitochondrial superoxide levels, reduced MnSOD cysteine glutathionylation, and ameliorates mitochondrial dysfunction. Together with alleviating F-actin oxidation via enhancing the antioxidant response (HO-1 and SOD1) and improved mitochondrial functions, GAT107 attenuates hyperoxia-induced macrophage phagocytic dysfunction. As such, GAT107 improves the bacterial clearance of PA lung infection and increases the rate of survival in hyperoxia-compromised mice.
4. RESULTS

4.1. Systemic administration of GAT107 increases survival in mice exposed to hyperoxia and challenged with PA lung infection
The prolonged exposure to hyperoxia is the critical mediator for the increased rates of mortality in patients with pulmonary infection (Chastre and Fagon, 2002; Rello, 2005; Spalding et al., 2017; M. Wang et al., 2019). To ascertain whether GAT107 administration to hyperoxia-compromised mice with *Pseudomonas aeruginosa* (PA) lung infection could improve animal survival rates, mice were initially exposed to 72 h of 95% O₂. During the 72 h of hyperoxia exposure, mice were administered intraperitoneally with GAT107 3.3 mg/kg or vehicle control as described previously above (section 2.2). As demonstrated in Figure 1, mice which remained in hyperoxic conditions had significantly higher mortality rates (20% of mice survived, p < 0.05) 24 h after challenge with bacterial lung infection when compared to mice that remained in room air (21% O₂) conditions (100% of mice survived). In mice administered with GAT107 (3.3 mg/kg), subjected to hyperoxic conditions, and challenged with PA lung infection, there was a significant improvement of mouse mortality rates (37.5% of mice survived, p < 0.05) when compared to hyperoxia control group. In addition, administration of GAT107 increased the mean survival time to 18.25 hours versus just 15.25 hours in the hyperoxia control group. Therefore, these data suggest that GAT107 improves animal survival of hyperoxia-compromised mice with PA lung infection.

4.2. Systemic administration of GAT107 improves bacterial clearance in mice exposed to 48 h hyperoxia and challenged with PA infection.
The prolonged exposure to hyperoxia can compromise the ability of subjects to clear bacterial lung infections (Patel et al., 2016, 2013; Sitapara et al., 2014). To determine if
GAT107 can restore the compromised clearance of bacteria in the lungs, we subjected mice to hyperoxia exposure, administered intraperitoneally (i.p.) of GAT107 3.3 mg/kg and instilled PA into lungs (i.e., bacterial challenge). As shown in Figure 2, mice that received prolonged exposure to 95% O₂ (i.e., hyperoxia) and were challenged with PA-induced lung infection (vehicle control group), had significantly higher levels of bacterial colonies in their airways (7.81 ± 0.24 log CFUs/mL, p < 0.0001) and lung tissue homogenate (7.68 ± 0.07 log CFUs/mL, p < 0.0001) compared to mice that remained at 21% O₂ (3.24 ± 0.15 log CFUs/mL in airways and 3.62 ± 0.35 log CFUs/mL in lung tissue homogenate). The mice that received 3.3 mg/kg i.p. of GAT107 (3.93 ± 0.52 CFUs/mL in airways and 4.49 ± 0.54 log CFUs/mL in lung tissue homogenate) had significantly lower levels of bacterial colonies in their airway fluids and lung tissue homogenate compared to the vehicle control group (****p < 0.0001). GAT107 administration had no significance difference when compared to room air control animals. These data indicate that GAT107 significantly decreases the lung bacterial burden in a mouse model of VAP.

4.3. GAT107 restores hyperoxia-compromised macrophage phagocytic function in a macrophage cell line and in primary macrophages
The decreased ability to clear bacterial lung infections in subjects compromised by hyperoxia exposure, is in part mediated by the impaired innate immune functions of macrophages. To determine whether GAT107 can restore hyperoxia-compromised macrophage function, RAW 264.7 and primary bone marrow-derived macrophages (BMDMs) were exposed to >95% O₂ (hyperoxia) for 24h and incubated with either 3.3 µM GAT107 or vehicle control (DMSO). The phagocytotic function of RAW 264.7 cells exposed to hyperoxia was significantly decreased (66.1 ± 2.5%, p < 0.0001)
compared to cells that remained at 21% $O_2$ (100 ± 0%). Hyperoxia-compromised phagocytosis was significantly attenuated by 3.3 μM GAT107 (86.3 ± 4.7%, p < 0.0001) compared to the vehicle control group (65.08 ± 2.53%) (Figure 3A and C). Furthermore, these effects were also replicated in primary BMDMs, where after exposure to 24h of hyperoxia, their phagocytic activity was significantly decreased (33.2 ± 5.3%, p < 0.0001), compared to macrophages that remained at 21% $O_2$ (100 ± 0%). GAT107 significantly increased the phagocytic activity of BMDMs exposed to hyperoxia (105 ± 13.9%, p < 0.001) compared to the vehicle control group (35.39 ± 3.9%) (Figure 3B and D). GAT107 incubation in hyperoxia-compromised macrophages showed no statistically significant difference when compared to cells that remain in room air control conditions. These results suggest that in vitro, GAT107 can attenuate the hyperoxic-induced decrease in macrophage phagocytic activity.

4.4. GAT107 Attenuates Hyperoxia-Induced Actin Oxidation and Alterations in Polymerization.
Exposure to macrophages with hyperoxia can result in oxidative post-translational modification to F-actin filaments that are critical for macrophage innate immune functions (Morrow et al., 2007; O'Reilly et al., 2003). To determine if GAT107 can attenuate hyperoxia-induced actin oxidation and altered actin polymerization, RAW 264.7 macrophages were exposed to 24 h of 95% $O_2$ (hyperoxia) and incubated with 3.3 μM GAT107. The fluorescent microscopic analysis and quantification of F-actin stress fiber formation, similar to previous studies (Morrow et al., 2007; O'Reilly et al., 2003), indicated that macrophages exposed to hyperoxia had significantly higher amounts of stress actin filament formation as indicated by an increased phalladoin/DAPI ratio (91.25 ± 1.09%, p < 0.0001), compared to cells that remained at room air (81.05 ±
Furthermore, 3.3 µM GAT107 significantly decreased stress actin formation produced by hyperoxia (83.45 ± 1.53%, p < 0.01), compared to the vehicle control group (89.53 ± 1.34%). GAT107 incubation to hyperoxia-compromised macrophages had no statistical difference when compared to cells that remained in room air control conditions. Furthermore, macrophages exposed to hyperoxia had increased total protein oxidation compared to the macrophages that remained in room air, and this was decreased by incubation with 3.3 µM GAT107 (Figure 4B). The proteins that migrated to approximately 43 kD were used as an indicator of actin oxidation. Based on the 43 kD marker proteins, we observed the same protein oxidation trend, suggesting that GAT107 also reduces the amount of hyperoxia-induced actin oxidation. Overall, these results indicate that GAT107 attenuates hyperoxia-induced alterations in actin polymerization by reducing the oxidation of total protein and actin.

4.5. The systemic administration of GAT107 attenuates hyperoxia-induced oxidative stress and increases the total lung antioxidant potential

Previous studies have shown that hyperoxia-induced oxidative stress mediates the decreased bacterial clearance functions of mice, which can be ameliorated when subjects are administered antioxidants, like ascorbic acid (Patel et al., 2016; Sitapara et al., 2020). To determine if GAT107 mitigates excessive lung oxidative stress in mice exposed to 95% O₂ (hyperoxia) and challenged with PA lung infection, the oxidative redox potential (ORP) and the lung antioxidant potentials (cORP) were determined. As shown in Figure 5, mice subjected to 95% O₂ had significantly higher oxidative-reduction potentials (298 ± 44.3 mV, p< 0.05) and lower antioxidant potentials (0.16
± 0.05 µC, p < 0.05), compared to mice that remained at 21% O₂ (204.26 ± 7.5 mV and 0.27 ± 0.03 µC, respectively). Indeed, the i.p. administration of 3.3 mg/kg GAT107 significantly attenuated the hyperoxia-induced increased lung oxidative-reduction potential (162.36 ± 22.1 mV, p<0.05), and increased the total antioxidant potential (0.47 ± 0.09 µC, p < 0.05), compared to the vehicle control group. GAT107 ameliorated hyperoxia-induced increased lung homogenate ORP to a level that showed no statistically significant difference when compared to animals that remained in room air control conditions. These data indicate that GAT107 attenuates lung oxidative stress by increasing the total antioxidant potential.

4.6. GAT107 Attenuates Hyperoxia-Induced Oxidative Stress by Restoring Hyperoxia-Compromised SOD1 Function

The prolonged exposure to hyperoxia induces oxidative stress and compromises the innate immune functions of macrophages, which can be attenuated by the treatment with antioxidants, like ascorbic acid and superoxide dismutase (Arita et al., 2007; Morrow et al., 2007; Patel et al., 2020). To determine if hyperoxia-induced oxidative stress is attenuated by GAT107, RAW 264.7 cells were exposed >95% O₂ (hyperoxia) and incubated with 3.3 µM GAT107. The prolonged exposure to hyperoxia significantly increased the total intracellular ROS levels (305.79 ± 20.21%, p < 0.0001), compared to macrophages that remained at 21% O₂ (100 ± 0%) (Figure 6A). GAT107 significantly decreased total intracellular ROS levels (231.05 ± 5.75%, p < 0.0001), compared to vehicle control (353.28 ± 27.81%) (Figure 6A). The prolonged exposure to hyperoxia significantly compromises SOD1 activity (45.59 ± 8.71%, p < 0.001), compared to cells that remained at 21% O₂ (100 ± 0%) when compared to 21% O₂ (Figure 6B). Hyperoxia-compromised SOD1 activity was significantly
restored in macrophages following treatment with 3.3 µM GAT107 (84.63 ± 10.75%, p<0.01), compared to vehicle control (41.56 ± 7.31) (Figure 6B). These results suggest that hyperoxia-induced oxidative stress is decreased by GAT107, in part, by the restoring the hyperoxia-compromised antioxidant activity of SOD1.

4.7. GAT107 Activates the Nrf2/HO-1 Antioxidant Pathway
Previous studies indicate that the activation of Nrf2 can protect mice against hyperoxia-induced acute lung injury and macrophage dysfunction by upregulating enzymes involved in the antioxidant, such as heme oxygenase-1 (HO-1) (Otterbein et al., 1999; Reddy et al., 2009; Wang et al., 2007). To determine if GAT107 can induce the activation of Nrf2, macrophages were exposed to 24h of 95% O₂ (hyperoxia) and incubated 3.3 µM GAT107. Our results indicate that under hyperoxic conditions (25.83 ± 7.19%), there was no significant increase in Nrf2 localization to the nucleus, compared to macrophages exposed to room air (21.2 ± 4.8%) (Figure 7A). However, GAT107 significantly increased Nrf2 nuclear localization (51.45 ± 4.08%, p <0.05), compared to vehicle control (31.03 ± 5.01%) (Figure 7A). Next, we determined the levels of the protein, HO-1 in macrophages by Western blot analysis. Under hyperoxic conditions (0.643 ± 0.075 AU HO-1/actin), there was a non-significant change in HO-1 levels when compared to 21% O₂ room air (0.107 ± 0.01 AU HO-1/actin) (Figure 7B). However, the incubation of macrophages with 3.3 µM GAT107 (2.38 ± 1.91 AU HO-1/actin, p < 0.0001) induced a significant increase in HO-1 levels, compared to vehicle control (0.745 ± 0.05 AU HO-1/actin) (Figure 7B). These results suggest that in vitro, GAT107 induces Nrf2 and HO-1 upregulation.
4.8. GAT107 attenuates hyperoxia-induced mitochondrial membrane hyperpolarization and mitochondrial superoxide accumulation

The exposure of macrophages to hyperoxia has been reported to produce mitochondrial damage (Ma Cui et al., 2018; Pagano and Barazzone-Argiroffo, 2003). Furthermore, mitochondrial damage has a significant inhibitory effect on macrophage phagocytosis (Orrenius et al., 2011). To determine if 24 h of hyperoxic exposure to macrophages affects mitochondrial function, the mitochondrial membrane potential was determined using the tetramethylrhodamine ethyl ester (TMRE) assay. Our results indicated that the mitochondrial membrane potential of macrophages exposed to hyperoxia was hyperpolarized (141 ± 13.8 %, p < 0.0001) compared to macrophages exposed to room air (100 ± 0%). Moreover, in this study, hyperoxia-induced mitochondrial hyperpolarization was significantly attenuated by GAT107 at 3.3 µM (62.85 ± 7.29 %, p < 0.0001) compared to vehicle control (123.5 ± 4.6%) (Figure 8A). Additionally, the prolonged exposure to hyperoxia also significantly increased the mitochondrial levels of superoxide (mitoSOX) (212 ± 23 %, p < 0.01) when compared to macrophages that remained in room air (21% O₂) conditions (100 ± 0%) (Figure 8B). Furthermore, GAT107 treatment significantly decreased (147.7 ± 33.4 %, p < 0.01) hyperoxia-induced increased mitoSOX levels when compared to hyperoxia-compromised macrophages treated with vehicle control (226.3 ± 31%) (Figure 8B). Therefore, these results suggest that GAT107 attenuates hyperoxia-induced mitochondrial membrane hyperpolarization along with the amelioration of hyperoxia-induced mitochondrial superoxide accumulation.
4.9. GAT107 Attenuates Hyperoxia-Compromised MnSOD Activity
Under normal cellular conditions, mitochondrial superoxide is rapidly dismutated into hydrogen peroxide by MnSOD (Candas and Li, 2014). To determine if hyperoxia-exposure affected total levels of MnSOD protein in macrophages, Western blot analysis was performed on whole cell lysates collected from RAW 264.7 cells exposed to 24 h of hyperoxia and incubated with 3.3 µM GAT107 (Figure 9A), as described previously. Interestingly, in macrophages exposed to hyperoxic conditions, there was a significant increase in the total levels of MnSOD protein (2.93 ± 0.18 MnSOD/actin, p < 0.001) when compared to cells that remained in room air control conditions (1.17 ± 0.23 MnSOD/actin). Furthermore, incubation with 3.3 µM GAT107 had no statistically significant effect (2.89 ± 0.13 MnSOD/actin) on hyperoxia-increased MnSOD protein levels when compared to the vehicle control group (2.78 ± 0.15 MnSOD/actin). Therefore, we postulated that the significant increase in mitochondrial superoxide following the prolonged exposure to hyperoxia could result from an alteration in the activity of MnSOD.

To determine if GAT107 modulated MnSOD activity under hyperoxic conditions, macrophage whole cell lysates were collected as previously described and subjected to a MnSOD enzyme activity kit assay. As shown in Figure 9C, macrophages exposed to hyperoxic conditions had a significant decrease in MnSOD activity (2.72 ± 0.11 U/mg protein, p < 0.0001) when compared to cells that remained in room air conditions (3.9 ± 0.2 U/mg protein). Interestingly, incubation with 3.3 µM GAT107 significantly increased the MnSOD activity of hyperoxia-exposed macrophages (3.26 ± 0.047 U/mg, p<0.001) when compared to macrophages treated with vehicle control.
(2.73 ± 0.09 U/mg) (Figure 9C). Therefore, these results suggest that GAT107 attenuates hyperoxia-compromised MnSOD function through modulating MnSOD activity independent of its whole cell protein levels.

4.10. GAT107 restores MnSOD activity by inhibiting hyperoxia-induced MnSOD glutathionylation

Previous studies indicate that under oxidative stress conditions, MnSOD undergoes post-translational modification and becomes less enzymatically active (Candas and Li, 2014). MnSOD activity was not dependent on whole cell MnSOD protein levels as GAT107 did not significantly alter MnSOD activity compared to the hyperoxia control group (Figure 10A). Therefore, we determined the effect of GAT107 on post-translational modification by the reversible glutathionylation of MnSOD, which decreases MnSOD activity. Western blot analysis of co-immunoprecipitated MnSOD indicated that macrophages exposed to hyperoxia had significant higher levels of glutathionylated MnSOD compared to those exposed to the room air control group (91.8 ± 6.7 % versus 7.6 ± 4.5 % GSH/MnSOD, p < 0.0001). Macrophages exposed to hyperoxia and incubated with 3.3 μM GAT107 had a significantly lower level of glutathionylated MnSOD compared to vehicle control (53.5 ± 7.5 % versus 91.2 ± 6.5 % GSH/MnSOD, p < 0.01). A correlation analysis indicated a significant negative correlation between an increase in the glutathionylation of MnSOD and MnSOD activity (correlation coefficient, r = -0.8818, p < 0.0001) (Figure 10B).
5. DISCUSSION
We have previously shown that treatment with the α7nAChR partial-agonist, GTS-21, can attenuate hyperoxia-compromised bacterial clearance functions in mice with PA lung infection (Sitapara et al., 2014), but does not significantly improve mouse survival rates.

In this study, we demonstrate that GAT107, an α7nAChR ago-PAM, significantly improves the survival rates of mice by attenuating hyperoxia-compromised bacterial clearance functions of mice. The improvement of bacterial clearance functions was caused by efficacy of GAT107 on attenuating hyperoxia-compromised macrophage functions. The hyperoxia-impaired macrophage functions were attenuated by the GAT107-mediated decreased oxidation of F-actin, a critical cytoskeletal component in macrophage phagocytosis. GAT107 protected mouse lungs and macrophages from the effects of hyperoxia by ameliorating hyperoxia-induced oxidative stress and enhancing the antioxidant response. These effects of GAT107 on macrophages were demarcated by improving the redox balance in mitochondria, resulting in attenuated mitochondrial stress and the enhanced antioxidant function of MnSOD. Interestingly, GAT107 modulated MnSOD activity via attenuation of hyperoxia-induced cysteine glutathionylation. These results suggest that GAT107 increases bacterial clearance by improving hyperoxia-compromised phagocytic function of macrophages via improving the redox balance of macrophages.

5.1. The systemic administration of GAT107 increases survival in hyperoxia-compromised mice with PA lung infection
Herein this study, we demonstrate that GAT107 administration significantly improves the survival rates of mice exposed to hyperoxia and then challenged with
PA lung infection. Since 1899, it has been previously reported that in subjects exposed to 100% oxygen tensions that it promotes the pathogenesis of pneumonia and lung injury (Smith, 1899). PA is a ubiquitous gram-negative bacterium, which is typically harmless, but in immunocompromised individuals, such as patients exposed to prolonged periods of hyperoxia, PA becomes highly opportunistic and pathogenic (Faure et al., 2018). The uncontrolled and elevated bacterial burden of PA in the lungs leads to disruption and damage to the endothelial and epithelial alveolar barriers, which can result in acute lung injury (ALI)/acute respiratory distress syndrome, leading up to multiple organ failure and death (Faure et al., 2018; Sadikot et al., 2005; M. Wang et al., 2019).

A key pathological feature characterizing hyperoxia-compromised subjects with PA lung infection is the presence of ALI. The prolonged exposure to hyperoxia itself can cause hyperoxia-induced acute lung injury (HALI) characterized by increased airway protein content and inflammatory cell infiltration, which is mediated in part by both oxidative stress and the extracellular accumulation of HMGB1 (Entezari et al., 2014; Kallet and Matthay, 2013; Patel et al., 2020; Sitapara et al., 2020). Previously, it has been demonstrated that activation of the cholinergic anti-inflammatory pathway can protect against shock-induced death and lung injury in dogs by reducing vasopermeability and TNF-α and IL-1 levels (Hu et al., 2014). Specifically, treatment with GTS-21 has been demonstrated to have a protective effect against both hyperoxia and LPS-induced lung injury through mitigating the pro-inflammatory response of macrophages (Sitapara et al., 2020, 2014; J. Wang et al., 2019). Therefore, based on the data presented in this study and others, the treatment of subjects that
receive hyperoxic oxygen therapy, such as those under mechanical ventilation, with activators of the cholinergic anti-inflammatory pathway like GAT107, may be effective in alleviating lung injury and subsequently improve survival rates. Importantly, length of mechanical ventilation days is directly associated with the incidence of mortality in ICU patients (Pranikoff et al., 1997). Thus, the development of treatments, like GAT107, that reduce pulmonary inflammation, lung permeability/edema, and lung injury, may be a potential therapeutic intervention to improve lung functions and allow clinicians to better use conservative oxygen strategies and/or reduce the amount of days a subject requires mechanical ventilation (Jaffal et al., 2017). However, future studies are needed to confirm whether the ability of GAT107 to increase the survival rates of hyperoxia-compromised mice with PA lung infection is through ameliorating inflammatory lung injury and modulating levels of HMGB1.

5.2. GAT107 Attenuates the Hyperoxia-Compromised Bacterial Clearance Functions of Mice with PA lung infection

As previously mentioned, high levels of PA in the lungs is damaging to pulmonary tissue, which can result in increased mortality rates in ventilated patients (Faure et al., 2018; Sadikot et al., 2005; M. Wang et al., 2019). As shown in Figure 2, mice subjected to the prolonged exposure to hyperoxia and challenged with PA lung infection have an impaired capacity to clear PA in the airways and lung tissue, and administration of GAT107 can significantly attenuate this dysfunctional host defense response. As compared to our previous findings where 4 mg/kg i.p. of GTS-21 was given three times a day under the same experimental paradigm (Sitapara et al., 2014), the twice daily administration of 3.3 mg/kg i.p. of GAT107 produced a ten-fold
improvement in the clearance of bacteria by the mice. Since agonists of α7nAChR are prone to receptor desensitization, we hypothesized that the ago-PAM properties of GAT107 may allow for lower and less frequent dosing to produce a similar magnitude of bacterial clearance, but a more detailed pharmacological evaluation will be required to verify our hypothesis. Nevertheless, these improved clinically relevant outcomes are important because as mentioned previously, patients receiving oxygen therapy will be exposed to hyperoxia for extended periods of times, which can compromise innate immune responses needed to clear bacteria, increasing the incidence of hospital-acquired infections (Kallet and Branson, 2016; Koenig and Truwit, 2006; Mach et al., 2011; Six et al., 2016). Moreover, other investigations have also utilized direct vagus nerve stimulation, which activates the cholinergic anti-inflammatory α7nAChR’s, and has been shown to improve the bacterial clearance functions and survival rates of subjects with sepsis (Borovikova et al., 2000; Huston et al., 2007; Kessler et al., 2012; Wang et al., 2016). Furthermore, the major cause of death in people with COVID-19 is fatal pneumonia mediated in part by a hyper-inflammatory response (Huang et al., 2020). Current expert opinions hypothesize that use of non-invasive vagus nerve stimulation may be a potential treatment strategy to mitigate the hyper-inflammatory syndrome in COVID-19 (Andersson et al., 2020; Staats et al., 2020). Therefore, the identification of novel therapeutic strategies, such as activators of the cholinergic anti-inflammatory pathway (e.g. GAT107, GTS-21, and vagus nerve stimulation), that restore host innate immune function to diminish the adverse effects of pneumonia are critically needed.
5.3. GAT107 Significantly Attenuates impaired Macrophage Innate Immune Functions produced by hyperoxia through mitigating the oxidation of F-Actin

Our results indicated that GAT107 significantly attenuated the hyperoxia-induced impairment of macrophage phagocytic function in cultured macrophages and primary BMDMs (Figure 3). Previously, we and others have reported that prolonged exposure to hyperoxia compromises macrophage phagocytic function (Morrow et al., 2007; Patel et al., 2013; Sitapara et al., 2014; Wang et al., 2015). Moreover, the partial-α7nAChR agonist, GTS-21 (5-50µM), has been shown to restore the impaired phagocytic function produced by hyperoxia, which was due, in part, to a decrease in HMGB1 release from macrophages (Sitapara et al., 2014). The GTS-21-mediated decrease in airway HMGB1 also improved bacterial clearance in the airways of mice challenged with *Pseudomonas aeruginosa* (Sitapara et al., 2014). Similar to our bacterial clearance results (Figure 2), GTS-21, at 25-50 µM (Sitapara et al., 2014), and GAT107, at 3.3 µM, had similar efficacy in significantly attenuating hyperoxia-induced phagocytic dysfunction of cultured macrophages, which as previously discussed, may be due to the ago-PAM properties of GAT107 which produces greater efficacy than the partial-agonism of α7nAChR by GTS-21. In addition, the direct stimulation of the vagus nerve has been previously shown increase the basal and sepsis challenged phagocytic activity of resident liver macrophages (Fonseca et al., 2019), which suggests that activation of macrophages α7nAChR may play a critical role in modulating intracellular pathways that mediate phagocytic activity.

Activation of neutrophil α7nAChRs with the direct agonist, nicotine, has been demonstrated to decrease the polymerization of F-actin (Huston et al., 2009).
Furthermore, macrophage phagocytic function is dependent upon the rapid polymerization of F-actin filaments for migration and phagocytosis of bacteria and to efferocytose apoptotic neutrophils (Capasso et al., 2016; Morrow et al., 2007; O'Reilly et al., 2003; Svitkina, 2018). However, previously, our lab and others have shown that the prolonged exposure to hyperoxia alone and in addition to PA infection, oxidizes actin filaments, resulting in dysfunctional actin polymerization (Morrow et al., 2007; O'Reilly et al., 2003). Antioxidants, such as procysteine and exogenous superoxide dismutase (SOD), protect against hyperoxia-induced disorganization of the actin cytoskeleton and attenuate phagocytic dysfunction (Morrow et al., 2007). As demonstrated in Figure 4, GAT107 significantly ameliorated hyperoxia-induced actin fiber disorganization by reducing the magnitude of protein oxidation. Protein oxidation of F-actin filaments may be due to macrophage activation, which increases superoxide production during the oxidative or respiratory burst response (Park, 2003). The respiratory burst response involves the assembly of NADPH oxidase enzymes that generate large amounts of superoxide, which kill the ingested pathogens (Park, 2003). However, macrophages exposed to hyperoxia for prolonged periods of time have significantly increased intracellular levels of superoxide (Freeman et al., 1982; O'Donovan and Fernandes, 2000; Pagano and Barazzone-Argiroffo, 2003). Consequently, hyperoxia exposure, in addition to activating respiratory bursts, may produce excessive oxidative stress, where high levels of ROS can induce the post-translational oxidation modifications of proteins such as actin (Chia et al., 2019; Rudyk and Eaton, 2014; van der Vliet et al., 2018). In addition, hyperoxia-compromised macrophages have compromised bactericidal functions to
kill ingested PA, which may be partly due to actin disorganization and subsequent
dysfunction to assemble bactericidal enzymes or phagolysosomes (Gore et al., 2020).
Overall, our results suggest that the efficacy of GAT107 to attenuate hyperoxia-
compromised macrophage function may be due, in part, to it decreasing the oxidation
of actin filaments.

5.4. GAT107 Ameliorates Oxidative Stress and Restores Antioxidant Functions
Next, we sought to evaluate whether protein oxidation in macrophages indicated the
possible presence of an oxidative-stress response in the lungs of hyperoxia-
compromised mice with PA lung infection. As demonstrated in Figure 5,
administration of GAT107 to mice decreases hyperoxia-induced increases in ORP by
increasing the total antioxidant potential in the lung tissue homogenate. In a 645-
person enrolled clinical trial for traumatic brain injury, a 20 mV increase in plasma
ORP levels was correlated with a 4-fold increase in mortality rates (Rael et al., 2009).
Conversely, a 1 unit increase in the inverse 1/cORP (antioxidant potential) was
correlated with 5-fold increase in mortality rates (Rael et al., 2009). Recently, we have
reported that in mice subjected to 72 h of hyperoxia, there was a significant increase
in lung lavage fluid ORP levels (Patel et al., 2020). Furthermore, 24 h of hyperoxia
exposure induces a significant increase in cultured macrophage lysate ORP levels
(Patel et al., 2016). Moreover, in both of the aforementioned studies, ascorbic acid
(50mg/kg i.p. or 1000 µM in cell culture) significantly decreased the hyperoxia-
induced increases in mouse lung lavage fluid and cultured macrophage lysate ORP
levels and significantly improved animal mortality rates after challenge with PA lung
infection (Patel et al., 2020, 2016).
As shown in Figure 6, similar to the results obtained in total lung homogenate, the prolonged exposure to hyperoxia increased total ROS levels in macrophages and also significantly decreased the antioxidant activity of SOD1. Our results demonstrated that treatment with GAT107 attenuated the hyperoxia-induced increase in macrophage ROS levels and also restored SOD1 activity. We hypothesize that GAT107’s activation of the α7nAChR may be restoring hyperoxia-compromised SOD1 activity by transcriptional upregulation and expression of SOD1 or by altering inhibitory post-translational regulatory modifications of SOD1 (Dinesh and Rasool, 2017). As discussed previously, prolonged exposure to hyperoxia and the activation of macrophages induces the production of high levels of ROS such as superoxide directly in the cytosol or indirectly by ROS leakage from the mitochondria into the cytosol. Normally, the major antioxidant in the cytoplasm that removes superoxide is SOD1 (Fukai and Ushio-Fukai, 2011; M. Fetherolf et al., 2017). Decreased SOD1 activity during hyperoxic conditions may therefore further affect the production and levels of cytoplasm superoxide. As such, the cytoplasmic abrogation of elevated ROS levels along with a compromised antioxidant defense system may then further participate and contribute to the oxidation of macromolecules such as F-actin. Although the exact amino acid residues in actin that are oxidized by hyperoxia remain to be determined, biochemical analysis and computational modeling suggest that NOX-mediated superoxide production and oxidation of actin F-actin filaments may occur at Cys10, 217, 257, 285, and 374 (Wilson et al., 2016).
5.5. GAT107 Activates Nrf2 and Upregulates HO-1
The GAT107-mediated restoration of the hyperoxia-induced redox imbalances may be due to its activation of the master antioxidant pathway, Nrf2 (Figure 7). Recently, we and others have shown that the activation of the Nrf2 pathway in macrophages in vitro and ex vivo in mice significantly increases innate immune functions and decreases lung injury (Cho et al., 2002; Patel et al., 2020; Reddy et al., 2009). The activation of α7nAChR by acetylcholine or GTS-21 results in downstream Nrf2 activation and the transcriptional upregulation of the genes that produce antioxidants such as glutathione (Hoover, 2017; Pagano and Barazzone-Argiroffo, 2003; Patel et al., 2017; Tsoyi et al., 2011; Zhang et al., 2019).

Heme oxygenase-1 (HO-1) is a Nrf2-regulated antioxidant that has been shown to decrease oxidative-induced lung injury (Cho et al., 2002; Choi and Alam, 1996; Reddy et al., 2009; Ryter and Choi, 2016). Under hyperoxic conditions, HO-1 null cardiomyocytes exhibit mitochondrial damage and decreased density, which may be due to heme toxicity (Suliman et al., 2017). Furthermore, hyperoxia exposure increases the number of hemoproteins and free reactive iron in lung tissues of mice (Dennery et al., 1998). It is likely that many of the free iron or heme groups found in these hyperoxia-compromised subjects are originated from cytochromes, cyclooxygenases, and other heme containing proteins found in the mitochondria (Soares and Hamza, 2016). The accumulation of free heme in macrophages results in heme-induced cell death characterized by damage to proteins, DNA, and lipid peroxidation (Vijayan et al., 2018)
HO-1 is the rate-limiting enzyme in heme metabolism, which results in the production of approximately 86% of all carbon monoxide (Ryter and Choi, 2013; Slebos et al., 2003). Furthermore, carbon monoxide produced by HO-1 has been shown to neutralize free iron and also activate anti-inflammatory pathways by downregulating pro-inflammatory cytokine production (Slebos et al., 2003; Soares and Hamza, 2016). Treatment with exogenous carbon monoxide protects against hyperoxia-induced endothelial cell apoptosis via exerting antioxidant functions and promoting the interaction of HO-1 with the pro-apoptotic factor, Bax (Wang et al., 2007). Interestingly, in hyperoxic-rat models, there is minimal transcriptional upregulation of HO-1 mRNA at 48h, and from 48h to 64h of continuous hyperoxia exposure, there is a steady-state increase in HO-1 mRNA (Choi and Alam, 1996; Lee et al., 1996). However, during this time, there is a substantial increase in rat mortality rates after 60 and 72h of continuous hyperoxia (Choi and Alam, 1996; Clerch and Massaro, 1993; Lee et al., 1996). In rats that received an HO-1 gene transfer and exposed to hyperoxia, there were significantly improved mortality rates, lung injury, and increased lung HO-1 mRNA and protein levels (Otterbein et al., 1999). In HO-1-deficient mice with sepsis, there was a decrease in the bacterial removal by macrophages, and this was attenuated by pretreating animals with carbon monoxide-releasing molecules (Chung et al., 2008). Conversely, it has also been shown in mice with the disrupted or inhibited expression of HO-1 and exposed to hyperoxia, that these mice are more protected from inflammatory lung injury and have decreased levels of total reactive iron in the lung (Dennery et al., 2003). Thus, the exact role that HO-1 plays in the lungs of mice and macrophages exposed to hyperoxia is not fully
understood, but the early induction of HO-1 by GAT107 may serve as a critical antioxidant involved in ameliorating hyperoxia-induced lung injury and improving the innate immune response in the bacterial clearance ability by macrophages.

5.6. Hyperoxia Disrupts Mitochondrial Membrane Integrity
As demonstrated in Figure 8A, mitochondrial membrane hyperpolarization occurs in macrophages exposed to 24 h of hyperoxia. Indeed, the hyperpolarization of mitochondrial membrane potential in T cells precedes terminal mitochondrial damage, resulting in cell death (Perl et al., 2004). Furthermore, mitochondrial hyperpolarization may indicate a dysregulation of oxidative phosphorylation, which increases ROS generation and the subsequent loss of ATP production (Chen et al., 2017; Pak et al., 2013, p. 20). The buildup of protons in the intermembrane space due to the inhibition of F_0F_1-ATPase proton motor functions can cause an electrochemical gradient shift resulting in a hyperpolarized mitochondrial membrane potential and the uncoupling of oxidative phosphorylation (Perl et al., 2004). As shown in Figure 8, hyperoxia-induced about a 41% increase in mitochondrial membrane polarization, and it has been reported that an increase of 28-32% mitochondrial membrane potential causes ETC uncoupling, disrupted ATP generation, and significant ROS generation (Perl et al., 2004; Skulachev, 1999). There are at least ten known sites of physiological ROS generation in the mitochondria (Lin and Beal, 2006; Orrenius et al., 2007; Starkov, 2008). However, it has been reported that in the presence of high levels of oxygen, the mitochondrial flavoprotein quinone oxidoreductase may be the sole cause of the increase in ROS levels (Hoffman and Brookes, 2009). In mice exposed to 72 h of hyperoxia, there is a significant inhibition of complex I activity (a known
cause of ROS generation) and decreased ATP production in isolated pulmonary mitochondria (Ratner et al., 2009). As demonstrated in this study (Figure 8A), GAT107 was able to significantly attenuate hyperoxia-induced macrophage mitochondrial hyperpolarization. Therefore, through normalizing the mitochondrial membrane potential, GAT107 is capable of mediating the maintenance of mitochondrial integrity and thus help establish conditions that resemble normal mitochondrial function.

5.7. GAT107 significantly attenuates the hyperoxia-induced decrease in MnSOD activity
Under normal physiological conditions, MnSOD catalyzes the dismutation reaction of mitochondrial superoxide into hydrogen peroxide at a rate of $2 \times 10^{9} \text{M}^{-1} \text{s}^{-1}$, which can be then quickly converted by catalase in water (Candas and Li, 2014; Ozden et al., 2011; Wang et al., 2018). However, as shown in Figure 9C, 24 h of hyperoxia exposure significantly decreased MnSOD activity. Other investigations have reported that the intermittent exposure of macrophages to hyperoxia significantly decreases MnSOD function at 7 days, but interestingly, after 3-weeks, MnSOD function is significantly increased (Kokubo et al., 2010). Conversely, guinea pigs exposed to hyperoxia for 3 days, alveolar macrophage MnSOD function was significantly increased (Aerts et al., 1995). However, in cultured macrophages overexpressing MnSOD or incubated with exogenous SOD, macrophages had significantly greater phagocytic capacity for PA after exposure to 24h of hyperoxia (Arita et al., 2007; Morrow et al., 2007).

In our study, GAT107 restored mitochondrial function as indicated by the normalization of mitochondrial membrane potential (Figure 8A). In addition, GAT107 also normalized mitochondrial superoxide levels (Figure 8B). The GAT107 - mediated
normalization of mitochondrial superoxide levels were also associated with the attenuation of hyperoxia-compromised MnSOD function (Figure 9B), suggesting that the generation of excessive mitochondrial superoxide may result from MnSOD dysfunction. Moreover, GAT107’s restorative effect of MnSOD function was not dependent on the overall protein levels of MnSOD (Figure 9A). Interestingly, in cultured macrophages exposed to 3-weeks of intermittent hyperoxia, macrophages survived only if they express significantly higher levels of MnSOD activity (Kokubo et al., 2010). Therefore, since GAT107 did not significantly alter the total protein levels of MnSOD, it suggests that MnSOD activity is not being modulated MnSOD protein levels. Thus, it is possible that GAT107 may be increasing MnSOD activity by post-translational modifications (Ho et al., 1996).

Indeed, it has been shown that post-translational modifications of MnSOD alters its enzymatic function (Candas and Li, 2014; Demicheli et al., 2018; Dikalova et al., 2017; Kim et al., 2017; Patil et al., 2013; Yamakura and Kawasaki, 2010). The glutathionylation of proteins has been reported to have a protective role against irreversible oxidative modifications (e.g. sulfonic acid) of reactive cysteine residues (Hurd et al., 2005; Janssen-Heininger et al., 2013). As shown in Figure 10, hyperoxia induces the glutathionylation of MnSOD and is significantly ameliorated by GAT107 (3.3 µM) treatment. Furthermore, the increase in MnSOD glutathionylation was significantly correlated with decreased MnSOD activity (Figure 10), demonstrating that glutathionylation of MnSOD modulates its enzymatic activity. Previously, it has been reported that under oxidative stress conditions, non-mitochondrial SOD1 is glutathionylated at Cys111 and FeSOD is glutathionylation at Cys57 (Candas and Li,
Currently, it remains to be determined how GAT107 decreases the glutathionylation of MnSOD. We postulate that GAT107 may be reducing MnSOD glutathionylation through pathways that augment the mitochondrial antioxidant capacity, thereby decreasing the oxidative burden on MnSOD by the GAT107-induced activation of the Nrf2/HO-1 pathway. It may be likely that the upregulation of HO-1 can cause preferential mitochondrial translocation. In human lung epithelial cells exposed to particulate matter 2.5 µm (PM2.5) collected from air pollution, it was determined that there was a significant increase in preferential transposition of HO-1 into the mitochondria, which may play a protective role in PM2.5 induced necrosis (Zhou et al., 2017). It has been previously reported that the activation of α7nAChR by acetylcholine or GTS-21 results in downstream Nrf2 activation and the transcriptional upregulation of antioxidants such as glutathione (Báez-Pagán et al., 2015; Hoover, 2017; Patel et al., 2017; Tsoyi et al., 2011; Zhang et al., 2019). It is likely that under oxidative stress conditions, both non-enzymatically and enzymatically catalyzed glutathionylation reactions occur in the mitochondria since the mitochondrial concentration of glutathione are approximately 5-10 mM (Hurd et al., 2005). Also, the de-glutathionylation of proteins may be facilitated by sulfiredoxins, glutaredoxin 2 (Grx2), and thioredoxin (Trx2) (Chia et al., 2019; Findlay et al., 2006; Janssen-Heininger et al., 2013). Thus, GAT107 could influence the de-glutathionylation of proteins by acting on one of these de-glutathionylating enzymes, although this remains to be determined. It may be likely that in hyperoxia-induced lung injury that there is dysregulation of proteins that maintain the glutathionylation profile of proteins since dysregulation of glutathionylation has been linked to lung
diseases such as idiopathic pulmonary fibrosis, asthma, and chronic obstructive pulmonary disorder (Chia et al., 2019).
6. CONCLUSIONS
As shown in Figure 11, the α7nAChR type 2 ago-PAM, GAT107, attenuates hyperoxia-induced innate immune dysfunction of mice with PA lung infection. GAT107 restored bacterial clearance functions of mice, in part, by mitigating hyperoxia-induced macrophage phagocytic dysfunction. Hyperoxia exposure also oxidized F-actin filaments in macrophages, which was reduced in cells incubated with GAT107. In mice exposed to hyperoxia with PA lung infection, there was a significant increase in lung oxidative stress and reduced antioxidant potentials that were attenuated by GAT107 administration. Indeed, in macrophages, GAT107 decreased the significant increase in intracellular ROS levels and loss of SOD1 antioxidant function produced by hyperoxia. GAT107 appeared to mediate the aforementioned effects of hyperoxia by activating Nrf2 and upregulating HO-1 expression. GAT107 attenuated the hyperoxia-compromised mitochondrial function and enhanced MnSOD function. Moreover, hyperoxia-induces MnSOD glutathionylation, which correlates significantly to decreased MnSOD function. Importantly, GAT107 reduces the amount of hyperoxia-induced MnSOD glutathionylation. Therefore, GAT107 restores mitochondrial function through modulating the glutathionylation status of MnSOD, which may participate in regulating oxidative stress-induced macrophage dysfunction.

Other studies have indicated that the inhibition of α7nAChR with antagonists (methyllycaconitine and α-bungarotoxin), only partially reduces GTS-21’s efficacy to inhibit LPS-induced secretion of IL-6 and TNFα (Garg and Loring, 2019). GTS-21 also partially ameliorates LPS-induced secretion of both IL6 and TNFα from cultured
macrophages where the a7nAChR gene was knocked out (Garg and Loring, 2019). In our study, we did not determine whether the efficacy of GAT107 was due to it selective activation of the α7nAChR. Thus, is it possible that GAT107 may interact with non-α7nAChR targets and future studies will be required to identify these targets. Therefore, regardless of GAT107’s mechanism of action, our results suggest that GAT107 may be a potential therapeutic candidate for the prevention and/or treatment of subjects with oxygen therapy - induced compromised immunity, including patients with VAP.
7. FUTURE DIRECTIONS

7.1. Exploring the efficacy of GAT107 on modulating hyperoxia-compromised mice and macrophages

A potential direction for future investigation is to further delineate the mechanisms underlying hyperoxia-induced mitochondrial dysfunction in macrophages. Connecting these features with ETC function, ATP production, and the role of redox imbalance via glutathionylation mechanisms is needed. Moreover, it is well established that lung protein glutathionylation plays a critical pathological role in the pathogenesis of lung diseases like asthma and idiopathic pulmonary fibrosis (Chia et al., 2019; Janssen-Heininger et al., 2013), however, it would be interesting to explore the role lung protein glutathionylation plays in hyperoxia-induced acute lung injury and macrophage dysfunction. Since this current study established that GAT107 induces an antioxidant response in mouse lung tissue and in macrophages, it would be interesting to explore what enzymes, such as glutathione S-transferase, glutaredoxin, thioredoxin, and peroxiredoxins, are involved in the glutathionylation or de-glutathionylation process of MnSOD and possible other proteins.

In these results, our lab has demonstrated that GAT107 can improve the bacterial clearance functions of mice with PA lung infection through alleviating hyperoxia-induced macrophage dysfunction (Figure 11). Recently, our lab has shown that GTS-21 can alleviate inflammatory lung injury in mice subjected to the prolonged exposure to hyperoxia (Sitapara et al., 2020). Increased lengths of hyperoxia exposure along with hyperoxia-induced inflammatory lung injury in combination with PA lung infection-induced lung injury contribute to poor prognosis’ and higher mortality rates in ventilated-associated pneumonia patients (Bassi et al., 2014;
Oliveira et al., 2014; Rello, 2005; Spalding et al., 2017; M. Wang et al., 2019). Thus, extending this study to determine if GAT107 ameliorates inflammatory lung injury is needed in order to fully ascertain the efficacy of GAT107 on improving survival rates in hyperoxia-compromised mice with PA lung infection. Indeed, inflammatory lung injury in hyperoxia-compromised subjects with and without PA lung infection can be attenuated via using neutralizing anti-HMGB1 antibodies to target extracellular HMGB1 in the airways of mice (Entezari et al., 2014; Patel et al., 2013). The accumulation of extracellular HMGB1 in the airways of mice is an important mediator of hyperoxia’s role in compromising alveolar macrophage functions. In addition, HMGB1’s unique pathological role may be both temporal and concentration dependent, yielding a striking and currently under explored pleiotropic role in the lung. Treatment with HMGB1 can directly cause macrophage dysfunction (Entezari et al., 2012) and can stimulate the endocytosis of HMGB1 into macrophages and both cause and contribute to a hyper-inflammatory response. Extracellular HMGB1 can augment pro-inflammatory responses of bacterial and viral pathogens (pathogen associated molecular patterns (PAMPS)) via the formation of HMGB1-PAMP conjugates (Andersson et al., 2020; Lu et al., 2014). These HMGB1-PAMP conjugates can then be endocytosed by macrophages and synergize the pro-inflammatory response, which may be a significant contributing factor underlying the cytokine-storm syndrome in COVID-19 (Andersson et al., 2020). Thus, it would be also beneficial to explore the role of GAT107 in ameliorating the secretion and extracellular accumulation of HMGB1 from macrophages in the context of treating inflammatory lung injury in oxygen-therapy treated patients, especially in COVID-19.
7.2. Can we use α7nAChR activators like GAT107 for the treatment of COVID-19?
The coronavirus disease of 2019 (COVID-19) caused by SARS-CoV2 is a complex
disease that has profound effects on the lung, central nervous system, circulatory
system's clotting mechanisms, cardiac tissue, and the gastrointestinal system
(Farsalinos et al., 2020b, 2020a; Rothan and Byrareddy, 2020). As of the writing of
this dissertation, the coronavirus pandemic has approximately 13,500,000 confirmed
cases and 9350,000 confirmed deaths worldwide. Current CDC estimates have
determined that 81% of patients with COVID-19 experience mild to moderate
symptoms including shortness of breath and mild forms of pneumonia (To et al.,
2020). In addition, between 14 and 5% of patients experience severe to critically
severe COVID-19 symptoms, respectively, which includes dyspnea, hypoxia, up to
respiratory failure and multiple organ failure (To et al., 2020). In the United States,
approximately 19% of all COVID-19 patients require hospitalization, of which, 6% are
admitted to Intensive Care Units (ICU) (Li et al., 2020). Although there is a wide range
of symptoms for COVID-19 patients, around 3 to 17% of all COVID-19 patients are
diagnosed with one of the hallmarks of severe COVID-19 (N. Chen et al., 2020; Guan
et al., 2020; Huang et al., 2020; Wang et al., 2020; Wu et al., 2020; Yang et al., 2020),
which is acute respiratory distress syndrome (ARDS) that results in the need for
patients to be mechanically ventilated as a life-saving intervention (Coperchini et al.,
2020). Ultimately, of the critically severe COVID-19 patients whom are admitted into
ICUs, there is an approximate 39 to 72% mortality rate (Huang et al., 2020; Wu et al.,
2020; Yang et al., 2020).
Early on, the clinical observations that studied COVID-19 disease progression determined that around 14% of all patients suffer from peripheral bilateral ground glass opacities by CT scan, which indicated that these patients were suffering with a partial airway filling of fluids and pulmonary wall thickening and also 75% of all patients have pneumonia (N. Chen et al., 2020). Moreover, this hallmark symptom of lung inflammation in severe COVID-19 has recently been hypothesized to be a condition known as the “cytokine-storm”, whereby the presence of excessive levels of pro-inflammatory cytokines exacerbate lung injury and promote multiple organ failure (Coperchini et al., 2020).

In this brief communication, the detailed mechanisms for SARS-CoV2 induced hypercytokemia are outside of the scope of this article, but we recommend reading Coperchini et al. (2020) for those concepts. Classically, the cytokine-storm syndrome is mediated in part by a hyperactive response of immunomodulatory molecules that results in high plasma levels of TNF-a, and interleukins including IL-1b, IL-8, and IL-6 (Lee et al., 2014). In the context of COVID-19, 5 separate study cohorts have reported that plasma levels of IL-6 range from 7 to 64 ng/mL in patients with severe disease phenotypes, which has previously been thought to be a major mediator of acute lung injury and ARDS (Sinha et al., 2020). However, as reported in 3 large cohort lung study groups (ALVEOLI, FACT, and SAILSS), non-COVID19 patients with diagnosed ARDS have plasma levels of IL-6 that range from 130 to 443 ng/mL in patients with a hypoinflammatory response and up to a 578 to 1618 ng/mL in patients with a hyperinflammatory response (Sinha et al., 2020). Although this disparity in the role in IL-6-mediated ARDS may be evident within patient plasma
samples compared between COVID-19 and large cohort ARDS studies exists, several therapeutics have been developed to target IL-6 as a COVID-19 treatment strategy. The monoclonal antibodies against IL-6, tocilizumab and sarilumab, are being used currently to treat the cytokine release syndrome for COVID-19 (clinical trials NCT04306705 and NCT04322773). Currently, due to the expedited clinical trial pipeline given to the development of these IL-6 targeted therapies and low clinical trial populations, the US FDA has still been unable to approve use of any IL-6 inhibitor for the treatment of COVID-19 (“Immune-Based Therapy | Coronavirus Disease COVID-19,” n.d.; Mehta et al., 2020). Internationally, clinicians are hopeful yet concerned about IL-6 therapies, due to the presence of several negative side effects. For instance, IL-6 inhibitors are noted for having the ability to increase the risk and rate of opportunistic infections (Calabrese et al., 2020). Moreover, clinically relevant models that simulate the use of oxygen therapy for prolonged periods of time, such as in those individuals receiving treatment for COVID-19, that the overexpression of IL-6 is protective against oxygen-induced lung injury in mice (Ward et al., 2000). Thus, it is still unclear what definitive role IL-6 is playing in COVID-19, which has been a catalyst for other investigations to continue searching for other possible therapeutic targets.

Recent epidemiological evidence shows that based on Chinese clinical trials, that smokers (N = 159 people) are statistically less likely to be hospitalized due to COVID-19 (total N = 1623 people) (Gonzalez-Rubio et al., 2020; Polosa and Caci, 2020). Moreover, non-peer reviewed clinical observations in France found that patients that are current smokers (<5% of all COVID-19 patients) are protected against SARS-CoV2
pathophysiology (Changeux et al., 2020). Although these studies and others have been steeped in hotly contested debate, the general conclusions definitively state that smoking tobacco is incredibly harmful, but perhaps the inhaled nicotine, with can be found in tobacco smoke, may have efficacious effects on ameliorating COVID-19 symptom severity. Indeed, nicotine as an anti-inflammatory molecule has been long recognized as an activating molecule of the cholinergic anti-inflammatory pathway (Pavlov et al., 2003; Scott and Martin, 2006).

Between the clinical data showing a possible link between COVID-19 disease severity and the exacerbated lung injury induced by SARS-CoV2 hyper-cytokemia (a.k.a. the cytokine-storm), COVID-19 has been postulated to be a disease of the cholinergic anti-inflammatory pathway (Andersson et al., 2020; Farsalinos et al., 2020a; Gonzalez-Rubio et al., 2020; Mazloom, 2020). The fundamental underpinning of this theory lies in the COVID-19-induced imbalance in control of cytokine secretion, which normally is maintained via vagus nerve mediated cholinergic signaling.

The cholinergic anti-inflammatory operates by the afferent vagus nerve sensing the presence of pathogens, damage-associated molecular patterns, and cytokines/chemicals. The afferent vagus nerve then signals to the central nervous system, and then communicates via the efferent vagus nerve to effector organs. At least in the lung, neuroendocrine cells receive effect vagus nerve innervations that form the parasympathetic nervous system relay (Wu et al., 2014). Activation of these pulmonary neuroendocrine cells found in the distal airways induces the release of acetylcholine, which can modulate the local inflammatory environment by binding to and activating the α7 nicotinic acetylcholine receptor (α7nAChRs) on alveolar
macrophages (Borovikova et al., 2000; Pavlov et al., 2003; Wang et al., 2003; Wu et al., 2014).

As previously mentioned, COVID-19 patients experiencing symptoms ranging from shortness of breath, hypoxemia, ARDS, and respiratory failure, receive some form of either non-invasive or invasive mechanical ventilation oxygen therapy. However, it is unclear whether the lung injury in COVID-19 patients is purely due to normal viral pathogenesis, patient induced lung injury (Fan et al., 2020), or exacerbated by the oxygen therapy itself. In clinically relevant laboratory models that simulate the use of oxygen therapy, the prolonged exposure to high concentrations of oxygen can directly cause injury to the lungs (Entezari et al., 2014; Patel et al., 2020; Ward et al., 2000). Moreover, these models of hyperoxia-induced inflammatory lung injury also can retain the cytokine-storm syndrome similar to what is observed in COVID-19 patients. In subjects with hyperoxia-induced inflammatory lung injury, the systemic administration of a small molecule called GTS-21, activates the α7nAChR-mediated cholinergic anti-inflammatory, which results in attenuated lung injury in mice (Andersson et al., 2020; Sitapara et al., 2020).

GTS-21 mediated the amelioration of inflammatory lung injury by significantly reducting both plasma and airway levels of the damage associated molecular pattern, high mobility group box-1 (HMGB1) (Sitapara et al., 2020). Importantly, increased plasma levels of HMGB1 have recently been shown to be significantly correlated with more severe clinical COVID19 outcomes that result in the increased ICU hospitalization and death rates (L. Chen et al., 2020). As such, use of therapeutics that activate the α7nAChR-mediated cholinergic anti-inflammatory pathway like nicotine
or GTS-21, that decrease the secretion of HMGB1 may be critical in preventing COVID-19 patients from severe disease complications.

HMGB1 as a target for treating COVID-19 has recently gained more attention. In other disease models, extracellular HMGB1 has been shown to form complexes with viral and bacterial pathogen, and damage associated molecules (Andersson et al., 2020; L. Chen et al., 2020). HMGB1 complexes are then endocytosed via RAGE receptors on innate immune cells, such as macrophages. Internalized HMGB1-complexes then can activate cytosolic receptors that activate inflammasome and cell death pathways (Andersson et al., 2020; Lu et al., 2014). It is hypothesized that HMGB1 can form complexes with SARS-CoV2 RNA fragments, which can then be up taken into innate immune cells to further exacerbate the host innate hyper-inflammatory response to SARS-CoV2 infection (Andersson et al., 2020). Moreover, activation of macrophage α7nAChR’s with small molecule agonists (acetylcholine and GTS-21), have been shown to inhibit the activation of inflammasomes via attenuating the endocytosis of HMGB1-LPS complexes into the cell (Lu et al., 2014; Yang et al., 2019).

Recent epidemiological studies have demonstrated that men and women have similar prevalence rates for COVID-19, but however, men are statistically more susceptible to severe COVID-19 symptoms and higher mortality rates (Jin et al., 2020). In pulmonary arterial hypertension patients, men have higher mortality rates, which was found to be to an increased level of pulmonary vascular necrosis and an increased amount of HMGB1 released into the extracellular milieu (Rafikov et al., 2019; Zemskova et al., 2020). Therefore, HMGB1 may be a possible contributing mechanism to explain why men with COVID-19 have higher mortality rates than compared to
women. Therefore, activation of the cholinergic anti-inflammatory pathway with α7nAChR activators has 2 hypothetical main modes of protection against SARS-CoV2 infection: 1) through the attenuation of SARS-CoV2 induced hyper-cytokemia, especially HMGB1, and by 2) inhibiting HMGB1-SARS-CoV2 RNA complexes that further exacerbate the hyper-inflammatory response.

In addition, *in silico*-based investigations have discovered a potential interaction between the α7nAChR and the SARS-CoV2 glycoprotein spike protein (Farsalinos et al., 2020b). Indeed, the interaction between α7nAChR and the spike protein was within the same magnitude as alpha-bungarotoxin, the snake venom derived molecule that selectively antagonizes the α7nAChR (Farsalinos et al., 2020b). Thus, it was hypothesized that this interaction may play a critical role in disrupting the cholinergic anti-inflammatory system for COVID-19 patients, and it may explain why severe COVID-19 patients present severe ARDS and lung inflammation.

Currently, there is conflicting data on whether nicotine’s role in modulating the expression of angiotensin 2 receptor (ACE2) is protective or harmful in SARS-CoV2 infection (C et al., 2020; Simons et al., 2020; Tindle et al., 2020). Depending on the organ and cell-type, nicotine has pleiotropic effects on ACE2 receptor expression (Leung et al., 2020; Tindle et al., 2020). The ACE2 receptor is the primary entry-point by which SARS-CoV2 infiltrates the cell. However, *in silico* molecular docking studies have revealed that nicotine may bind to ACE2 and competitively inhibit the ACE2-SARS-CoV2 complex (C et al., 2020). Therefore, although nicotine may have a potential role in mediating SARS-CoV2 cell invasion, nicotine has a well-established potent anti-inflammatory property that may be useful in the treatment or prevention
of COVID-19 associated ARDS. Nicotine, under the strict supervision and guidance of a physician, may then therefore be a potential therapeutic strategy in conjunction with palliative care, anti-virals, antibiotics, and antibodies for COVID-19, if used under heavily controlled conditions.

Given the wide availability commercially for nicotine containing products, their affordable manufacturing cost, and a range of delivery routes that can be tailored to meet individual patient needs, nicotine has then been thought to be a likely potential treatment in conjunction with anti-virals, antibodies, and antibiotics for the treatment of COVID-19. As a disclaimer, the use of tobacco products is known to cause cancer and increases the susceptibility to infections, so only under the strict supervision of a physician-guided treatment strategy should nicotine ever be potentially considered for the treatment of any disease.
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