2019

PREVENTION OF CRYSTALLINE SILICA-INDUCED INFLAMMATION BY THE ANTI-MALARIAL HYDROXYCHLOROQUINE

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PREVENTION OF CRYSTALLINE SILICA-INDUCED INFLAMMATION BY THE ANTI-MALARIAL HYDROXYCHLOROQUINE

By

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Thesis

presented in partial fulfillment of the requirements
for the degree of

Master of Science
in Toxicology

The University of Montana
Missoula, MT

May 2019

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Abstract

Burmeister, Rachel, M.S., May 2019 Toxicology

Prevention of Crystalline Silica-Induced Inflammation by the Anti-Malarial Hydroxychloroquine

Chairperson: Andrij Holian

Exposure to inhaled crystalline silica ($cSiO_2$) is common in occupations where there is cutting, milling, or grinding of $cSiO_2$ containing material. The Occupational Safety and Health Administration estimates that over 2 million workers may be exposed to inhaled $cSiO_2$ in the United States. Inhalation of $cSiO_2$ causes acute and chronic inflammation and may lead pulmonary diseases such as silicosis, as well as an increased risk of developing autoimmune diseases. Unfortunately, treatment of $cSiO_2$-induced lung diseases is limited and primarily focused on supportive care.

Inflammation caused by $cSiO_2$ begins when, $cSiO_2$ particles are phagocytized by alveolar macrophages. Interaction between the particle and lysosomal membrane results in damage to the phagolysosomal membrane; a state known as lysosomal membrane permeability (LMP). Leakage of lysosomal contents into the cytoplasm induces NLRP3 inflammasome activation leading to cell death and systemic inflammation. There are currently no pharmaceutical treatments that are directed at this mechanism of disease. Many existing pharmaceuticals become sequestered in the lysosome through an ion-trapping mechanism, and our laboratory aims to determine if these pharmaceuticals are capable of blocking permeabilization of the lysosomal membrane. Previously, our laboratory has shown that the tricyclic antidepressant, imipramine, blocks inflammatory cytokine production and toxicity in alveolar macrophages after exposure to $cSiO_2$. The objective of this research is to determine whether another pharmaceutical, hydroxychloroquine, prevents $cSiO_2$-induced toxicity by blocking LMP in alveolar macrophages. The ability to target the mechanism responsible for initiating particle-induced inflammation may lead to potential treatments and prevention strategies for people exposed to $cSiO_2$.

Acknowledgements

I would like to express my thanks to the Center for Environmental Health Sciences for giving me the opportunity to obtain quality scientific training. I would like to thank my mentor Dr. Andrij Holian and the other members of my committee, Dr. Scott Wetzel and Dr. Chris Migliaccio, for your guidance and valuable insight. Thank you to the research staff Britt Postma, Mary Buford Pam Shaw, Lou Herritt, Fred Rhoderick, and Ray Hamilton for all the time spent teaching me the techniques that allowed me to conduct this research. I would also like to thank the rest of the faculty and staff in the Department of Biomedical and Pharmaceutical Sciences for sharing your knowledge and experience. To all of the graduate students, thank you for your friendship and encouragement.

I am profoundly grateful for the support of my family and friends, both near and far, for their unwavering faith in me, the generosity with which they listened, and their unfailing kindness.

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List of Key Abbreviations

AM: Alveolar macrophages

BMDM: Bone marrow derived macrophages

$cSiO_2$: Crystalline silica

ELISA: Enzyme-linked immunosorbent assay

HCQ: Hydroxychloroquine

IL: Interleukin

LMP: Lysosomal membrane permeabilization

LPS: Lipopolysaccharides

OSHA: Occupational Safety and Health Administration

PBS: Phosphate buffered saline buffer

SSC: Side-scatter

MFI: Mean fluorescent intensity

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Chapter 1: Background and Significance

1.1 Silica Exposure

Exposure to inhaled crystalline silica particles ($cSiO_2$) remains a significant occupational health concern. The Occupational Safety and Health Administration (OSHA) estimates that over 2 million workers may be chronically exposed to inhaled $cSiO_2$ in the United States alone (Bang et al., 2015). Lung disease caused by $cSiO_2$ inhalation was recognized as an occupational hazard as far back as 1690 when Hippocrates observed that miners often developed difficulty breathing (Greenberg, Waksman, & Curtis, 2007). However, it is now known that $cSiO_2$ exposure is associated with both pulmonary and systemic disease including pulmonary fibrosis, silicosis, chronic inflammation, and autoimmune diseases (Pollard, 2016).

Materials containing $cSiO_2$ are used for purposes ranging from building materials to electronic components (IARC, 2012). Exposure is most common in occupations such as mining, construction, and manufacturing where $cSiO_2$ containing materials are mechanically disturbed creating inhalable aerosolized particles less than 10 μm in size (Leung, Yu, & Chen, 2012; Pollard, 2016). While the use of control measures, including dust control and wet-milling practices, have been successful at reducing the cases of silicosis in the United States and other developed countries, overexposures happen regularly and low level exposures over long time-frames are common (Bang et al., 2015; Castranova & Vallyathan, 2000). Modern technologies are also creating new opportunities for exposure. Silica nanoparticles have become the most widely used nanotechnology and are being used extensively for biomedical purposes (J. Wang et al., 2017). In the developing world, $cSiO_2$ exposure is more common due to a lack of regulation and use of personal protection equipment. Additionally, the demographics of $cSiO_2$ exposed populations in

developing countries may be markedly different from developed nations. In the United States the majority of people employed in trades where $cSiO_2$ exposure is likely have historically been men, and between 2001-2010 over 95% of silicosis cases in the United States were male. In developing countries women and children make up a significant portion of $cSiO_2$ exposures due to the increased likelihood of these individuals being employed in unskilled work (Brass et al., 2010; Sharma, 2008; Tiwari, Saha, & Parikh, 2009).

Pulmonary morbidities associated with the deposition of $cSiO_2$ in the lungs include bronchitis, silicosis, chronic obstructive pulmonary disease, increased risk of tuberculosis infections, and cancer. Additionally, $cSiO_2$ exposure is associated with development of systemic diseases including rheumatoid arthritis, scleroderma, systemic lupus erythematosus, and chronic renal disease (Leung et al., 2012; Pollard, 2016). This research focuses on the mechanisms following $cSiO_2$ exposure that lead to silicosis. Silicosis is a fibrotic disease characterized by pulmonary edema, chronic interstitial inflammation, and the development of silicotic nodules (Kawasaki, 2015). Silicosis is classified into several subtypes determined by the amount of $cSiO_2$ inhaled and length of exposure period. Acute silicosis, also called silicoproteinosis, occurs after exposure to high levels of $cSiO_2$ for a short period of time. During acute silicosis the alveolar spaces become filled with lipid and protein rich fluid and extensive neutrophilic infiltration occurs. Patients experience severe symptoms due to decreased gas exchange capabilities and respiratory failure (Castranova & Vallyathan, 2000). Chronic silicosis develops after 15-20 years of low to moderate $cSiO_2$ exposure and is the most commonly seen form of silicosis due to occupational exposures. An accelerated form of chronic silicosis can occur after 5-10 years of higher exposure levels (Pollard, 2016).

After inhalation, $cSiO_2$ particles small enough to enter deep into the lung reach the alveolar spaces; however, cellular mechanisms for clearing debris from the alveolar spaces are ineffective at removing $cSiO_2$ particles. When the resident alveolar macrophages (AM) attempt to phagocytize and clear the particles, the $cSiO_2$ causes caspase-1 dependent pyroptosis of the AM (Bergsbaken, Fink, & Cookson, 2009). This propagates a chronic inflammatory response as subsequent AM ingest the particles and continue a cycle of cell death. The damaged cells release damage-associated molecular patterns (DAMPs) and inflammatory cytokines such as TNF-α, IL-1β, and IL-6 recruiting other inflammatory cells, primarily other macrophages and polymorphonuclear neutrophils, and stimulating fibrogenic processes (Beamer & Holian, 2007; Kawasaki, 2015; Pollard, 2016; Rimal, Greenberg, & Rom, 2005). Fibroblasts surround the $cSiO_2$ particles with collagen creating fibrotic nodules which increase in size as disease progresses. The nodules can become necrotic and massive fibrosis develops throughout the lungs. The resulting damage can be severe and compromise pulmonary function (Rimal et al., 2005).

1.2 The NLRP3 Inflammasome and Lysosomal Membrane Permeability

Inflammation induced by $cSiO_2$ is directed by the NLRP3 inflammasome which drives the production of the inflammatory cytokines IL-1β and IL-18 (Jo, Kim, Shin, & Sasakawa, 2016; Sayan & Mossman, 2016). Two signals are required for the induction of the NLRP3 inflammasome (Fig. 1). The first signal is a priming signal which upregulates expression of the NLRP3 components. It is dependent on activation of the NF-κB pathway through TLR signaling due to various pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) (Biswas, Hamilton, & Holian, 2014; Sayan & Mossman, 2016). The

translocation of NF-κB to the nucleus also increases transcription of pro-IL-1β and pro-IL-18. A second signal induces the assembly of the NLRP3 inflammasome into the multi-protein complex containing the apoptosis-associated speck-like protein (ASC) which binds to procaspase-1. Procaspase-1 is cleaved into an active form and in turn cleaves pro-IL-1β and pro-IL-18 into their mature, secreted forms (Hornung et al., 2008). Together, the first signal and the second signal induce an inflammatory cascade and ultimately cell death (Bunderson-Schelvan et al., 2016).

There are several mechanisms proposed to initiate the second signal. These include potassium efflux, mitochondrial damage, reactive oxygen species production, and lysosomal injury; however, the primary mechanism of $cSiO_2$-produced NLRP3 induction is thought to be lysosomal injury (Jo et al., 2016). When $cSiO_2$ particles are phagocytized by AM, they interact with the interior of the phagolysosomal membrane and cause disruption to the membrane. This is referred to as lysosomal membrane permeability (LMP) (Bunderson-Schelvan et al., 2016; Serrano-Puebla & Boya, 2016). The compromised lysosomal membrane allows the acidic hydrolases, including cathepsin B, normally contained by the lysosome to leak into the cytosol (P Boya & Kroemer, 2008). There are currently no pharmaceuticals that target a molecular mechanism of $cSiO_2$ toxicity to reverse or prevent silicosis; however, targeting the lysosome in order to block LMP could be a promising way to diminish $cSiO_2$ toxicity (Biswas et al., 2017).

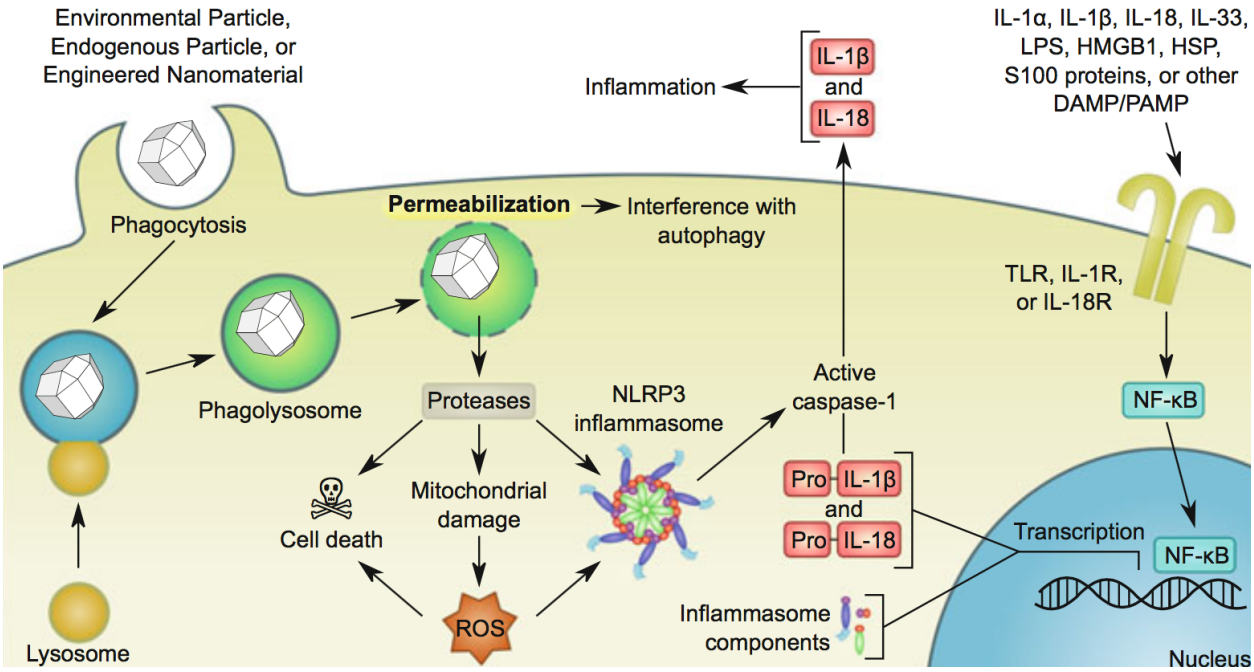

Figure 1: Proposed model of LMP

Model of the inflammatory cascade following particle-induced lysosomal membrane permeability. (Bunderson-Schelvan et al., 2016)

1.3 Cationic Amphiphilic Drugs

Many existing pharmaceuticals are known to interact with and become sequestered in the lysosome. This primarily occurs in a class of drugs known as cationic amphiphilic drugs (CAD). CAD tend to be weak bases that easily diffuse through membranes in their unprotonated state. Once they have diffused into the lysosome, they become protonated in the acidic environment subsequently preventing them from diffusing out of the lysosome. This ion-trapping mechanism results in a high concentration of drug stored within the lysosome (Villamil Giraldo et al., 2014). While this is considered an off-target effect for many drugs, this action may provide an opportunity to prevent particle-induced damage. Previously, our laboratory has shown that one CAD,

imipramine (IMP), can reduce pulmonary inflammation after exposure to $cSiO_2$ both *in vivo* and *in vitro* (Biswas et al., 2017). IMP is a tricyclic antidepressant that has been shown to have some protective effects against lung injury (Yang et al., 2010). Interestingly, another CAD, hydroxychloroquine (HCQ) is commonly used in the treatment of the autoimmune disease systemic lupus erythematosus (Fig. 2). Exposure to $cSiO_2$ has been shown to contribute to the development of systemic lupus erythematosus; however there has been no research examining the effect of HCQ on $cSiO_2$-induced toxicity.

HCQ has been extensively used to prevent malaria and as treatment for autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (Olsen, Schleich, & Karp, 2013). Recently HCQ has been investigated for use in diseases including endometriosis, cancer, cardiovascular disorders, diabetes, and infectious diseases (Ben-Zvi, Kivity, Langevitz, & Shoenfeld, 2012; D. Chen et al., 2018; Ruiz et al., 2016). There are many proposed mechanisms to explain how HCQ could be acting in such a diverse number of diseases. Some evidence has shown that HCQ can reduce inflammatory cytokine production by macrophages and alter macrophage phenotype (D. Chen et al., 2018). Other reports show changes in B and T cell signaling and inhibition of phospholipase A2 (Ben-Zvi et al., 2012). HCQ's effect on cancer and endometriosis have been attributed to modulation of autophagy. Furthermore, in a murine model of endotoxic shock, Chen *et al.* showed that chloroquine can decrease activation of the NLRP3 inflammasome and block production of IL-1β and IL-18 (D. Chen et al., 2018). However, no work has been done to examine whether HCQ accomplishes this by preventing destabilization of the lysosomal membrane nor if HCQ can prevent $cSiO_2$-induced LMP.

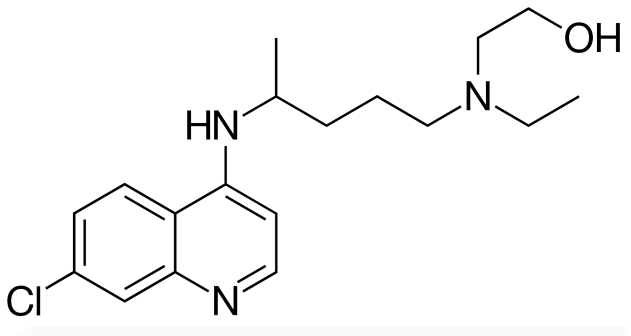

Figure 2: Chemical structure of HCQ

IUPAC name 2-[4-[(7-chloroquinolin-4-yl)amino]pentyl-ethylamino]ethanol

1.4 Specific Aims

The objective of this research was to determine if HCQ reduces $cSiO_2$ toxicity in alveolar macrophages. There are currently no pharmaceuticals that target a molecular mechanism of $cSiO_2$ toxicity to reverse or prevent silicosis; however, targeting the lysosome in order to block LMP could be a promising way to diminish $cSiO_2$ toxicity (Biswas et al., 2017). The objective of this research is to determine if HCQ has the ability to attenuate pulmonary $cSiO_2$ toxicity. My hypothesis is that HCQ blocks LMP and decreases $cSiO_2$-induced inflammation in alveolar macrophages.

Specific Aim 1: Determine that $cSiO_2$-induced toxicity is blocked by hydroxychloroquine *in vitro.*

Hypothesis: LMP will be decreased in HCQ treated alveolar macrophages which are exposed to $cSiO_2$ particles in vitro. In vitro studies will be conducted in order to show that HCQ can prevent LMP after $cSiO_2$ exposure. These studies will be conducted in bone marrow derived macrophages

(BMDM) from C57Bl/6 mice. LMP will be assessed through a digitonin assay measuring β-N-acetylglucosaminidase release. Additionally, inflammasome activation, toxicity, and cytokine production will be measured.

Methods:

Toxicity Assays

Using C57Bl/6 BMDM, I will show that HCQ blocks $cSiO_2$-induced toxicity. BMDM will be exposed to various doses (0-50 μg/mL) of $cSiO_2$. Toxicity will be analyzed with a common tetrazolium viability (MTS) assay (Promega, cat. G3580) and the lactate dehydrogenase assay (Promega, cat. G1780). NLRP3 inflammasome activation will be confirmed by assaying the release of IL-1β (R&D Systems, cat. DY201).

Measures of LMP

- In order to determine if HCQ can reduce LMP caused by $cSiO_2$, LMP will be quantified through measurement of released α-N-acetylglucosaminidase after digitonin extraction based on Aits *et al.,* 2015, as previously described by our laboratory (Aits, Jäättelä, & Nylandsted, 2015; Jessop, Hamilton, Rhoderick, Shaw, & Holian, 2016)
- Alternative methods of assessing LMP using Lysotracker dyes will also be used.

Phagocytosis

To ensure that LMP is not being altered due to differences in phagocytosis after being treated with HCQ, the relative amount of particle phagocytized by macrophages will be assessed by measuring relative side-scatter by flow cytometry of untreated macrophages and those treated with HCQ.

Specific Aim 2: Establish that HCQ prophylactically reduces $cSiO_2$-induced inflammation in a murine model of acute silicosis.

Hypothesis: Mice treated with HCQ before $cSiO_2$ treatment will show reduced toxicity after acute $cSiO_2$ exposure. C57Bl/6 mice will be treated with HCQ by oral gavage (to simulate normal pharmaceutical administration of HCQ by patients) for 7 days prior to oropharyngeal instillation of $cSiO_2$. Twenty-four hours after $cSiO_2$ exposure mice will be sacrificed. Inflammatory cytokines, cathepsin release, and toxicity will be assessed.

Methods: Mice will be treated with HCQ (10 mg/kg) through oral gavage once per day for a duration of seven days. Mice will then be exposed to $cSiO_2$ (0.25 mg or 1 mg) by oropharyngeal instillation and sacrificed 24 hours after exposure. Whole lung lavage fluid will be collected and assayed for cathepsin release as a measure of LMP. Lavage fluid will be assessed for protein, inflammatory cytokines (IL-1β, TNF-α, IL-6, IL-18, IL-33), cytotoxicity, and infiltration of immune cells.

Chapter 2: Methods

2.1 Mice

C57Bl/6 mice (Jackson Laboratories, Bar Harbor, ME) were housed in the University of Montana's specific-pathogen-free Laboratory Animal Resources facility. The mouse room is maintained on a 12 hr light/dark cycle and mice were provided with mouse feed and deionized water *ad libitum*. Male and female mice between 8-16 weeks of age were used for the studies. Euthanasia was administered through intraperitoneal injection of a lethal dose of pentobarbital sodium (Euthasol, Virbac, Fort Worth, TX). The University of Montana Institutional Animal Care and Use Committee (Missoula, MT, USA) approved all procedures performed on the animals.

2.2 Crystalline Silica

Crystalline silica (Min-U-Sil-5, average particle size 1.5-2 μm in diameter) was obtained from Pennsylvania Sand Glass Corporation (Pittsburgh, PA), and acid washed in 1N HCl. The $cSiO_2$ was washed with sterile water four times and dried in an oven at 200°C. Before use, $cSiO_2$ particles were suspended in PBS or dispersion media (for *in vivo* experiments) and sonicated for at least 2 min (550 watts at 20 kHz) by a cup-horn sonicator in a circulating water bath (Misonix, Inc. Farmingdale, NY, USA). Dispersion media consisted of PBS containing 0.6 mg/ml mouse serum albumin and 0.01 mg/ml 1,2-dipalmitoyl-sn-glycero-3-phosphocholine.

2.3 Isolation and Culture of Bone Marrow Derived Macrophages

Bone Marrow Derived Macrophages (BMDM) were generated as described previously (Migliaccio, Buford, Jessop, & Holian, 2008; Pfau et al., 2004). C57Bl/6 mice were sacrificed and the hind legs were removed. Complete media (RPMI, 10% FBS, Penicillin/Streptomycin) was washed through the femur and tibia to collect the bone marrow cells. Cells were incubated in T75

flasks at a density of $3.0x10^7$ overnight at 37°C for stromal elimination by adherence. The next day, nonadherent cells are transferred to a new flask at a density of $1.5x10^7$ cells per flask. Macrophage colony stimulating factor (M-CSF) (20 ng/mL R&D Systems) was added and cells were spiked with M-CSF (10 ng/ml R&D Systems) every 3-4 days. BMDM were used on day 10.

2.4 *In Vitro* Toxicity Assays

BMDM were platted in a flat-bottom, tissue culture- treated 96-well plates at 1×10^5 cells/well in 100 μL of RPMI complete media and incubated with lipopolysaccharides (LPS) (20 ng/mL) for inflammasome priming. Cells were treated with 25 μM of hydroxychloroquine sulfate (Sigma-Aldrich cat. H0915-5MG), 25 μM imipramine hydrochloride (Sigma-Aldrich cat. 10899-5G), or 100 nM bafilomycin (EnzoLife Sciences cat. BML-CM110-0100) for 30 minutes prior to addition of $cSiO_2$. Cells were exposed to various doses (0-50 μg/mL) of $cSiO_2$ and plates were incubated in a 37°C water-jacketed CO_2 incubator (ThermoForma, Houston, TX) for 24 hours. Toxicity induced by $cSiO_2$ was determined by two complementary assays, a lactate dehydrogenase assay (Promega, cat. G1780) and a common colorimetric tetrazolium viability (MTS) assay (Promega, cat. G3580), and read on a plate reader (Molecular Devices SpectraMax M4 colorimetric microplate reader). In order to avoid artifacts in the optical density values, the MTS reaction was transferred to a clean plate to separate it from the cell/particle mixture adhered to the plate bottom. Data were normalized to a percent relative to the no particle, no HCQ control cells. NLRP3 inflammasome activation was assayed by measuring the release of IL-1β by using a commercially available ELISA kit (R&D Systems, cat. DY201).

2.5 *In Vivo* Treatments

C57Bl/6 mice were treated with either hydroxychloroquine (Hydroxychloroquine Sulfate, Calbiochem cat. 509272) reconstituted in PBS (10 mg HCQ/kg/per day) or vehicle (PBS) by oral

gavage once a day for seven days. After the seven days, mice were instilled with 1 mg or 0.25 mg of $cSiO_2$ through oropharyngeal aspiration. Prior to gavage and instillation of $cSiO_2$, mice were briefly anesthetized by isoflurane. For instillation, the sedated mouse is positioned vertically while 50 μL of dispersion media with or without $cSiO_2$ was dispensed with a pipette to the back of the throat. By holding the tongue to the side, the mouse could not swallow and, therefore, aspirated the volume into the lungs. Mice were sacrificed 24 hours after $cSiO_2$ exposure for analysis by collecting whole lung lavage fluid. The lungs and heart were removed from the chest cavity, and 1 mL of cold PBS was washed in and out of the lungs four times in order to collect concentrated lung fluid. Samples were centrifuged at 400 x *g* for 5 minutes and the supernatant was saved for analysis. The lungs were lavaged with an additional 4 mL of PBS in order to collect the maximum number of cells. Lavage cells were counted using a Coulter Z2 particle counter (Beckman Coulter, Brea, CA, USA) and resuspended in RPMI 1640 culture media supplemented with 10% fetal bovine serum, sodium pyruvate, and an antibiotic-antimycotic solution (Mediatech, Manassas, VA) for differential analysis or flow cytometry. Cells were stained for differential analysis with a Wright-Geimsa stain in a Hematek 2000 autostainer (Miles-Bayer-Siemens Diagnostics, Deerfield, IL, USA).

2.6 *In Vivo* Lung Injury and Inflammation Assays

Toxicity was measured in the lung lavage fluid by assaying for LDH as above. Cytokines from lung lavage fluid were measured by a multiplex immunoassay (MSD U-PLEX, Meso Scale Diagnostics, U-PLEX, cat K15069L-2) because of the sensitivity and small sample size used. Seven cytokines IFN-γ, IL-1β, IL-6, IL-10, IL-13, IL-33, and TNF-α were included in the 7-plex plate used for this experiment. Protein in the lung lavage fluid was assayed by the Pierce BCA Protein Assay Kit (ThermoFisher Scientific cat. 23225) following manufacturers specifications.

Extracellular cathepsin B release in the lavage fluid was analyzed as previously described by our laboratory (Sager et al., 2016). In a 96 well plate, 50 μL of whole lung lavage was combined with 50 μL of a cathepsin B inhibitor (Calbiochem cat. 219385) or 50 μL PBS and incubated at room temperature for 15 min. Then the cathepsin substrate Z-LR-AMC (specific to cathepsin B, cathepsin L and cathepsin V; R&D systems cat. ES008) at 20 μL in PBS was added to 50 μL of whole lung lavage fluid in a total reaction volume of 150 μL. The assays were incubated at 37°C for 1 hour then fluorescence was measured using a plate reader at 380 nm excitation and 460 nm emission. The wells with the only the cathepsin substrate measured the total (cathepsin B, cathepsin L and cathepsin V) cathepsin activity in the lavage fluid while wells with the cathepsin B inhibitor measured the remaining cathepsin L and cathepsin V activity. By subtracting the inhibitor wells from the total cathepsin well, the level of cathepsin B was calculated.

2.7 *Ex Vivo* Culture for IL-1β Production

Cells collected during the whole lung lavage of mice treated with 0.25 mg of $cSiO_2$ *in vivo* were plated for *ex vivo* IL-1β culture. Lung lavage fluid was centrifuged and the supernatants saved for other analyses. Cells were resuspended in 250 μL of complete media, counted, and then 100 μL of cell suspension was plated in a 96 well plate. Cells were primed for inflammasome activity with LPS (20 ng/mL) and incubated for 24 hours at 37°C. After incubation, supernatants were collected and assayed with a commercial IL-1β kit (R&D Systems, cat. DY201). Data were normalized to IL-1β release per $1x10^5$ AM using the total cell count and percent of macrophages in the whole lung lavage.

2.8 Flow Cytometry

Flow cytometry was used to assess phagocytosis of $cSiO_2$ particles and lysosomal uptake of LysoTracker dye both in BMDM and AM collected in the lung lavage of treated mice. Internalization of $cSiO_2$ was determined by using a side-scatter technique previously described (Hamilton, Thakur, Mayfair, & Holian, 2006). BMDM or lung lavage cells were cultured or collected as described above. BMDM were treated with 25 μM HCQ for 30 minutes and then exposed to 50 μg/mL $cSiO_2$ for 4 hours using 1.5 mL microfuge tubes and end over end tumbling (Lab Quaker Shaker, Thermo Forma). Lung lavage cells had no additional treatment of HCQ or $cSiO_2$ after the *in vivo* exposures. Both lung lavage cells and BMDM were treated with LysoTracker Red DND-99 (ex/em 577⁄590 nm, ThermoFisher Scientific cat. L7528) at 50 nM for 30 min. The cells were centrifuged, resuspended in PAB, and transferred to filter-top flow cytometry tubes (BD Biosciences, San Jose, CA) for analysis. Phagocytosis and LysoTracker uptake data were expressed as mean fluorescent intensity. Cells were analyzed on a Life Technologies Attune NxT Acoustic Focusing Cytometer with the YL-1 585/16 nm laser.

2.9 Lysosome Membrane Permeabilization Assay

Lysosome membrane permeabilization (LMP) was assessed using methods modified from Aits *et al*. (Aits et al., 2015) and as described previously by our laboratory (Jessop, Hamilton, Rhoderick, Fletcher, & Holian, 2017). BMDM were plated in 24 well plates at a density of 2×10^5 cells per well. Cells were treated with or without HCQ (25 μM) and with or without $cSiO_2$ (50 μg/mL). Cells were washed twice with PBS and placed on ice. BMDM were then incubated with 200 μL of cytosol extraction buffer, which consisted of 250 mM sucrose, 20 mM Hepes, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 0.5 mM pefabloc (Sigma-Aldrich cat. 76307-100mg), pH 7.5, and digitonin (15 μg/mL) (Sigma-Aldrich cat. D141-100MG), for 15 min on ice with

rocking. The concentration of digitonin for optimal extraction of the cytosolic fraction was determined by titration. β-N-acetylglucosaminidase (NAG) activity was measured by adding 30 µL cytosolic extract to 100 µL of NAG reaction buffer (0.2 M sodium citrate, pH 4.5 with 300 µg/mL 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (Sigma-Aldrich cat. 37067-30-4) and assessed on a plate reader (20 min; 45s intervals; 356 nm excitation; 444 nm emission). Extracted cytosolic LDH activity was measured as described above and used as a control to which the NAG activities were normalized.

2.10 Statistical Analysis

Depending on the data type, a parametric one or two-way analysis of variance (ANOVA), followed by *post hoc* mean comparison (Holms-Sidak etc.) was used throughout the research described. Statistical significance will be defined as a two-tailed probability of type I error at less than 5% ($p < 0.05$) unless otherwise stated. The minimum number of experimental replications was 3.

Chapter 3: Results

3.1 HCQ decreases $cSiO_2$ toxicity *in vitro*

The ability of HCQ to decrease toxicity and cell death *in vitro* was assessed using C57Bl/6 BMDM. BMDM were primed for inflammasome activation with LPS (20 ng/mL) and then treated with either HCQ (25 μM), imipramine (IMP, 25 μM), or bafilomycin A1 (BAF, 100 nM) for 30 min prior to $cSiO_2$ exposure. IMP and BAF were positive controls for blocking $cSiO_2$-induced toxicity. Bafilomycin A1 (BAF) specifically inhibits vATPases to prevent lysosomal acidification, which previous studies have indicated is necessary for particle induced toxicity (Jessop et al., 2017). In biological systems small particles develop a protein shell called a protein corona. The protein corona is degraded in the acidic environment of the lysosomes which allows the particle to directly interact with the lipid membrane causing LMP. Preventing acidification is thought to prevent degradation of the protein corona and protect the lysosome from LMP (F. Wang et al., 2013). Imipramine, a tricyclic anti-depressant, was previously shown by our laboratory to block $cSiO_2$-induced toxicity; therefore, it was used as a comparison to HCQ (Biswas et al., 2017).

Cells were exposed to 0, 50, or 100 μg/mL of $cSiO_2$ and incubated for 24 hrs at 37° C. HCQ, IMP, and BAF were all found to block toxicity at both the 50 μg/mL and 100 μg/mL exposures of $cSiO_2$ as determined by the LDH assay and MTS assay (Fig. 3). No toxic effect from either HCQ or IMP alone was found in cells not exposed to $cSiO_2$. Protective effects were more pronounced at the 50 μg/mL $cSiO_2$ exposure as there was significant cell death at the 100 μg/mL $cSiO_2$ dose which could not be completely overcome by the HCQ, IMP, or BAF. Cell supernatants were assayed for IL-1β as a representative measure for NLRP3 inflammasome activation. IL-1β production was drastically decreased in exposed cells receiving HCQ, IMP, and BAF (Fig. 4).

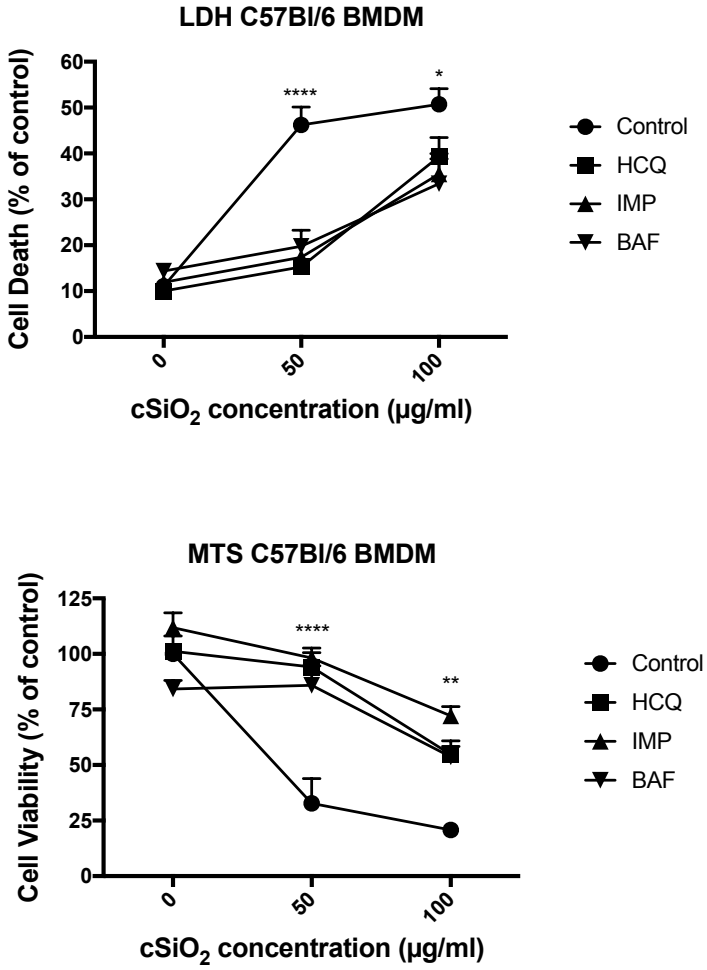

Figure 3: HCQ decreases $cSiO_2$-induced toxicity in BMDM.

BMDM were treated with HCQ (25 µM), IMP (25 µM), or bafilomycin A1 (100 nM) and exposed to 0, 50, or 100 µg/mL of $cSiO_2$. HCQ, IMP, and BAF caused statistically significant decrease in $cSiO_2$ toxicity as determined by decreased LDH release and increased cell viability.

(**** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$) n = 3-4

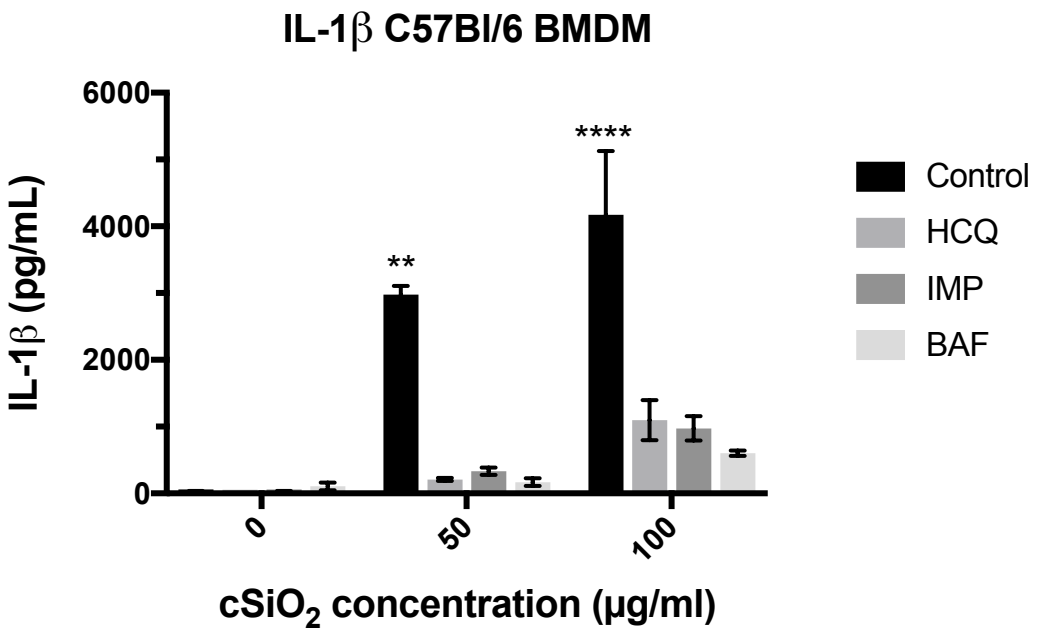

Figure 4: HCQ blocks the production of IL-1β in BMDM exposed to $cSiO_2$.

Cell supernatants were assayed for IL-1β as a representative measure for NLRP3 inflammasome activation. IL-1β production was blocked in exposed BMDM treated with HCQ (25 μM), IMP (25 μM), or bafilomycin A1 (100 nM) at both 50 (** $p < 0.01$) and 100 μg/mL (**** $p < 0.0001$) $cSiO_2$ exposures. Symbols indicate significance between control and treated cells. n = 3-4

3.2 HCQ does not affect $cSiO_2$ uptake *in vitro*

In order to confirm that HCQ was not blocking $cSiO_2$ toxicity by reducing phagocytosis of the $cSiO_2$ particles, the amount of $cSiO_2$ taken up by the BMDM was quantified by flow cytometry (Fig. 5). BMDM which have taken up $cSiO_2$ into their phagolysosomes have higher side-scatter (SSC) due to the deflection of laser light by the $cSiO_2$ particles. BMDM exposed to $cSiO_2$ (50 μg/mL) with or without HCQ (25 μM) had significantly higher side scatter (****$p < 0.0001$) than BMDM not exposed to $cSiO_2$. There was no difference between the SSC of control vs HCQ-treated cells nor $cSiO_2$ vs HCQ + $cSiO_2$ cells. This indicates that HCQ does not affect the ability of BMDM to take up $cSiO_2$.

Additionally, BMDM were also treated with LysoTracker Red DND-99 at 50 nM for 30 min (as per manufacturers recommendations) to assess the relative acidity of the lysosomes (Fig. 6). HCQ treatment caused a decrease in the amount of LysoTracker taken up by lysosomes. This finding suggests that HCQ is present in the lysosomes and may cause lysosomes to be less acidic. In turn, this may help protect the lysosomes from being permeabilized by $cSiO_2$. Cells treated with HCQ or HCQ + $cSiO_2$ had significantly less (*$p < 0.05$) LysoTracker uptake than control cells or cells exposed $cSiO_2$ without HCQ treatment. Together, the *in vitro* results collected in these experiments are consistent with HCQ being able to reduce LMP after $cSiO_2$ exposure.

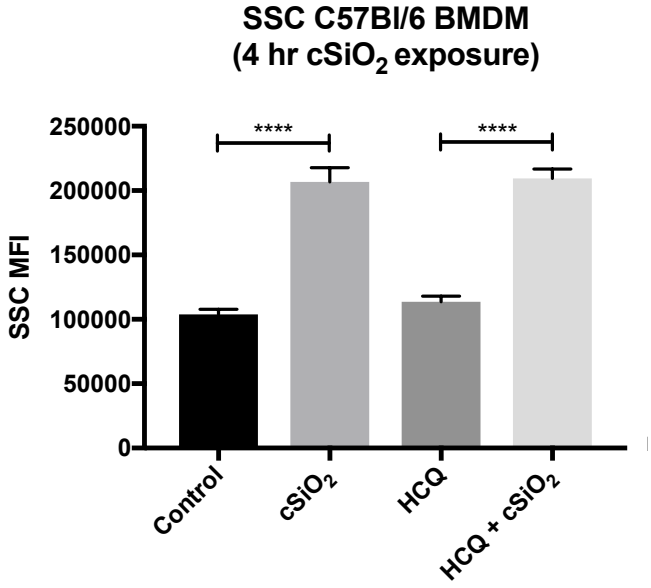

Figure 5: HCQ does not affect uptake of $cSiO_2$ *in vitro*.

BMDM which have taken up $cSiO_2$ into their phagolysosomes have higher side-scatter (SSC). BMDM exposed to $cSiO_2$ ± HCQ had significantly higher side scatter (****$p < 0.0001$) than BMDM not exposed to $cSiO_2$. There was no difference between the SSC of control vs HCQ-treated cells nor $cSiO_2$ vs HCQ + $cSiO_2$ cells; therefore, HCQ does not affect the ability of the BMDM to phagocytize $cSiO_2$ particles. $n = 3$

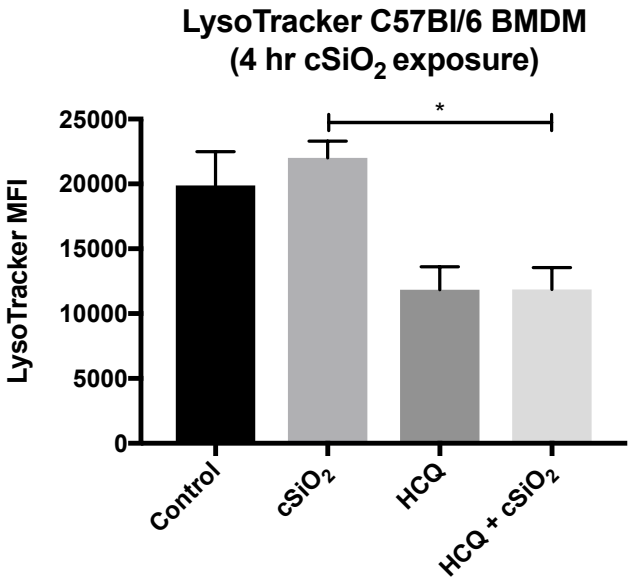

Figure 6: HCQ reduces the uptake of LysoTracker *in vitro*.

BMDM were treated with LysoTracker Red DND-99 at 50 nM for 30 min to assess the relative acidity of the lysosomes. HCQ treated cells showed less uptake of LysoTracker. Cells exposed to $cSiO_2$ without HCQ treatment had significantly (*$p < 0.05$) more LysoTracker uptake than cells exposed to $cSiO_2$ and treated with HCQ. $n = 3$

3.3 HCQ blocks LMP *in vitro*

Direct quantification of the ability of HCQ to block LMP *in vitro* was assessed through a method modified from Aits *et al*. which measures the release of β-*N*-acetyl-glucosaminidase (NAG) into the cytosol after cell permeabilization by the detergent digitonin (Aits et al., 2015). NAG is a lysosomal hydrolase which is not present in the cytosol unless the lysosomal membrane has been compromised. Using BMDM the level of digitonin is titrated so that the cell membrane is permeabilized while the lysosomal membrane remains intact. The lysosomal membrane contains less cholesterol than the cell membrane making the digitonin required to permeabilize the lysosomal membrane higher than that required for the cell membrane (Aits et al., 2015). BMDM were treated with or without HCQ (25 μM) and with or without $cSiO_2$ (50 μg/mL) and incubated for 4 or 24 hours. Cytosolic LDH activity was measured as described above and used as a control to which the NAG activities were normalized. HCQ was able to significantly block the release of NAG from lysosomes after both 4 ($*p < 0.05$) and 24 ($***p < 0.001$) hours. This shows that HCQ blocks $cSiO_2$-induced LMP *in vitro.*

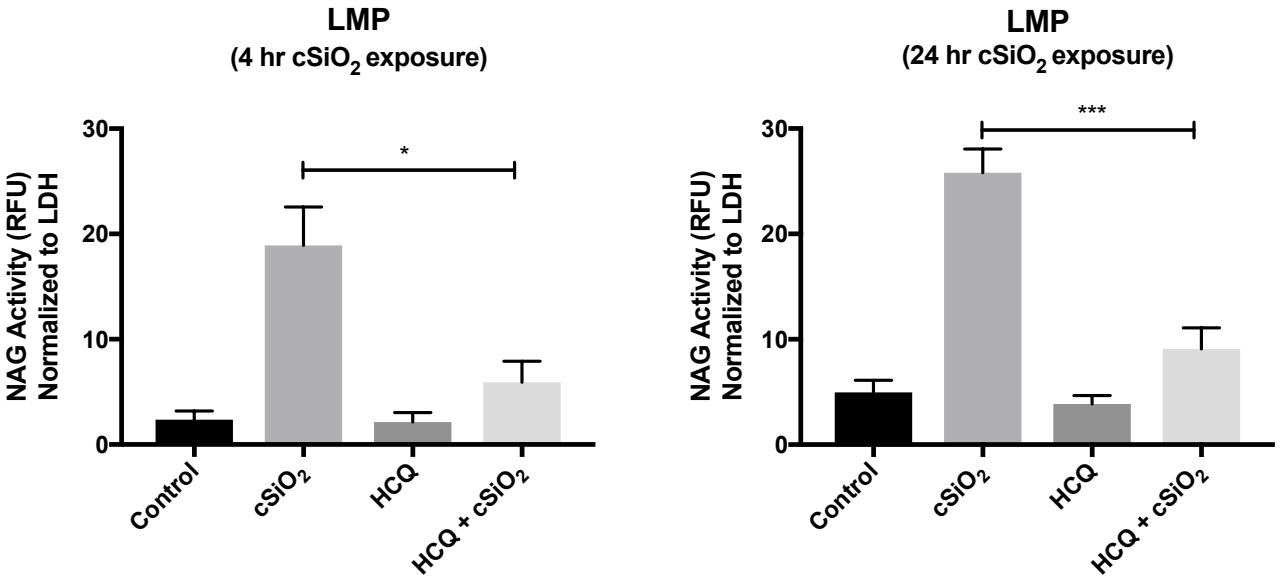

Figure 7: HCQ blocks LMP *in vitro*.

BMDM were treated with or without HCQ (25 μM) and with or without $cSiO_2$ (50 μg/mL) and incubated for 4 or 24 hours. HCQ was able to significantly block the release of NAG from lysosomes after both 4 (*$p < 0.05$) and 24 (***$p < 0.001$) hour $cSiO_2$ exposures. $n = 3$

3.4 Evaluation of HCQ on $cSiO_2$ uptake *in vivo*

The above *in vitro* studies demonstrated that HCQ accumulated in macrophages and blocked the ability of $cSiO_2$ to cause LMP and downstream events including toxicity and NLRP3 inflammasome activation as determined by IL-1β release. However, *in vivo* studies have not been previously conducted in order to determine whether the *in vitro* studies predict *in vivo* outcomes. Therefore, C57Bl/6 mice were treated with HCQ at 10 mg/kg/day or PBS by oral gavage for 7 days. This dose of HCQ was chosen based on previous *in vivo* studies with HCQ (X. Chen et al., 2017; Gómez-Guzmán et al., 2014). At the end of the 7-day pre-treatment period, mice were exposed to either 0.25 mg or 1 mg of $cSiO_2$ by oropharyngeal instillation (in 50 μL of dispersion media). Control mice received only dispersion media. Mice were sacrificed 24 hours post $cSiO_2$ exposure. The 1 mg of $cSiO_2$ exposure, has been commonly used in previous studies allowing comparison to previous results (Biswas et al., 2017). A lower dose of 0.25 mg was also evaluated. Naïve mice generally have around $3\text{-}4x10^5$ AM residing in their lungs. Therefore, the 0.25 mg dosage more closely matches the *in vitro* dose used in this study of 50 μg/mL $cSiO_2$ per $1x10^5$ BMDM.

In order to investigate whether HCQ was incorporated into the AM during the 7-day HCQ *in vivo* treatment, AM from whole lung lavage were treated with LysoTracker Red DND-99 and the cells analyzed by flow cytometry. As in the *in vitro* experiments, AM from mice treated with HCQ showed significantly less uptake of the LysoTracker dye. This finding suggests that in mice treated with HCQ at 10 mg/kg by oral gavage for 7 days did have HCQ sequestered into lysosomes of the AM (Fig. 8). Additionally, in order to rule out that HCQ was reducing toxicity due to changes in $cSiO_2$ uptake by the AM *in vivo*, side scatter data of the whole lung lavage cells were collected by

flow cytometry. Side scatter was increased as expected in the $cSiO_2$-treated mice and there was no difference between the control and HCQ-treated mice nor the $cSiO_2$ and HCQ + $cSiO_2$-treated mice. This matches the *in vitro* data showing that changes in $cSiO_2$-induced toxicity in HCQ-treated mice is not due to a change in the ability of the AM to phagocytize the $cSiO_2$ (Fig. 9).

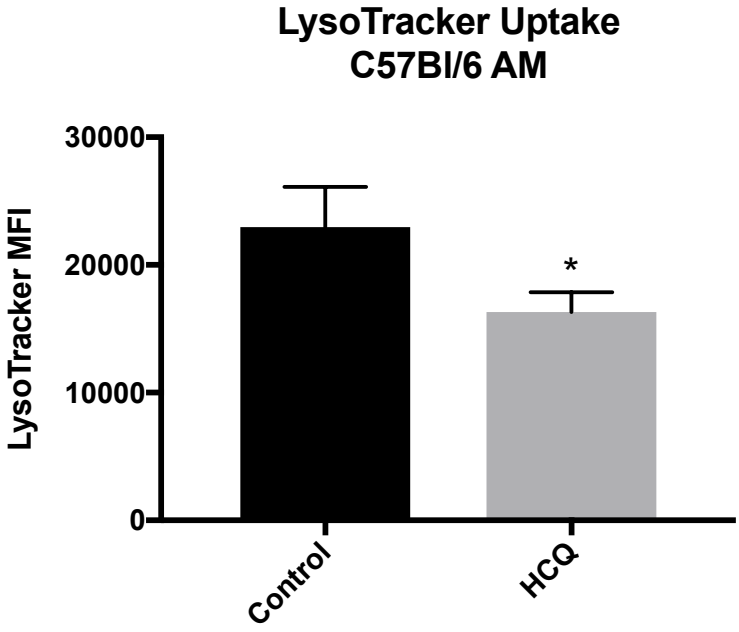

Figure 8: HCQ accumulates in lysosomes of AM after treatment by oral gavage.

AM from whole lung lavage of control or HCQ treated C57Bl/6 mice were incubated with LysoTracker Red DND-99 for 30 minutes prior to analysis by flow cytometry. In AM from mice treated with HCQ, there was significantly less uptake of the LysoTracker dye (*$p < 0.05$). This result is consistent with HCQ being sequestered into the lysosomes of the AM *in vivo*. $n = 4$

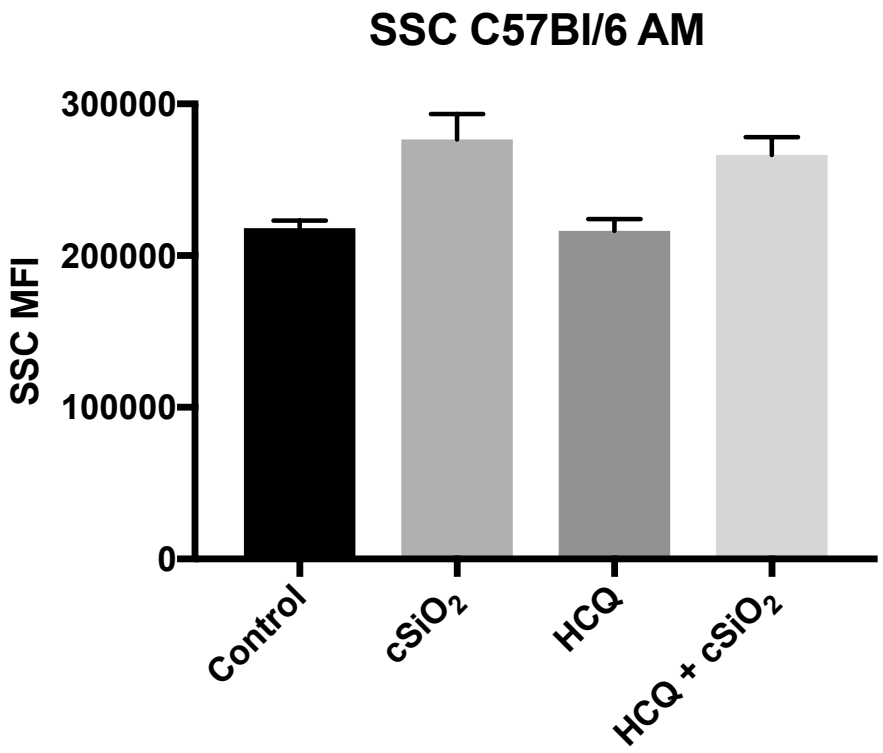

Figure 9: HCQ does not affect phagocytosis of $cSiO_2$ by AM *in vivo*

Whole lung lavage cells from mice treated *in vivo* ± HCQ and ± $cSiO_2$. Side scatter was increased as expected in the $cSiO_2$-treated mice. There was no difference between the control and HCQ-treated mice nor the $cSiO_2$ and HCQ + $cSiO_2$-treated mice suggesting that a difference in the uptake of $cSiO_2$ was not contributing to changes in toxicity. *n = 4*

3.5 Evaluation of HCQ on $cSiO_2$-induced inflammation and toxicity *in vivo*

Instillation of $cSiO_2$ is known to cause lung inflammation and infiltration of immune cells. Therefore, differential cells counts were conducted on the whole lung lavage fluid (Fig. 10). At the 1 mg $cSiO_2$ dose, there were no differences in the percentage of cells or the raw cell numbers between the HCQ treated mice and control mice ± $cSiO_2$ exposure. Mice exposed to $cSiO_2$ displayed the expected neutrophilic infiltration. At the 0.25 mg $cSiO_2$ dose, $cSiO_2$-exposed mice which were treated with HCQ had a significant increase in the percentage of macrophages and a significant decrease in the percentage of neutrophils recruited to the lungs as compared to $cSiO_2$ exposed mice with no HCQ treatment. Additionally, $cSiO_2$-exposed mice with HCQ treatment had a greater number of macrophages in their lungs 24 hours after $cSiO_2$ exposure than did $cSiO_2$ exposed mice without HCQ treatment. This suggests that macrophages may be protected from $cSiO_2$ induced pyroptosis with HCQ treatment.

In order to evaluate lung injury following instillation of $cSiO_2$, lavage supernatants were assayed for LDH release, total protein (bicinchoninic acid assay, BCA), relative total cathepsin, and relative cathepsin B levels (Fig. 11). Although not significant, LDH, total cathepsin, and cathepsin B release were all lower in HCQ treated mice at both the 0.25 mg and 1 mg $cSiO_2$ exposures. Total area under the LDH curves for control and HCQ treated mice were 241.3 ± 100.6 and 165.1 ± 52.58 respectively (Table 1). Surprisingly, LDH appeared to be higher in the 0.25 mg $cSiO_2$ exposed mice than the 1 mg $cSiO_2$ exposed mice, relative to control mice. While data were normalized to control mice, the 0.25 mg $cSiO_2$ exposure and the 1 mg $cSiO_2$ experiments were conducted at different times and the LDH assays run on different days which could account for this apparent discrepancy. Total protein in the lung lavage supernatants, as measured by a BCA

assay, was used to assess lung injury. The BCA assay showed a slight decrease in the amount of protein in the lung lavage fluid at the 1 mg $cSiO_2$ dose in HCQ-treated animals versus control. As expected the lung protein level increased as the $cSiO_2$ dose increased in both control and HCQ-treated mice.

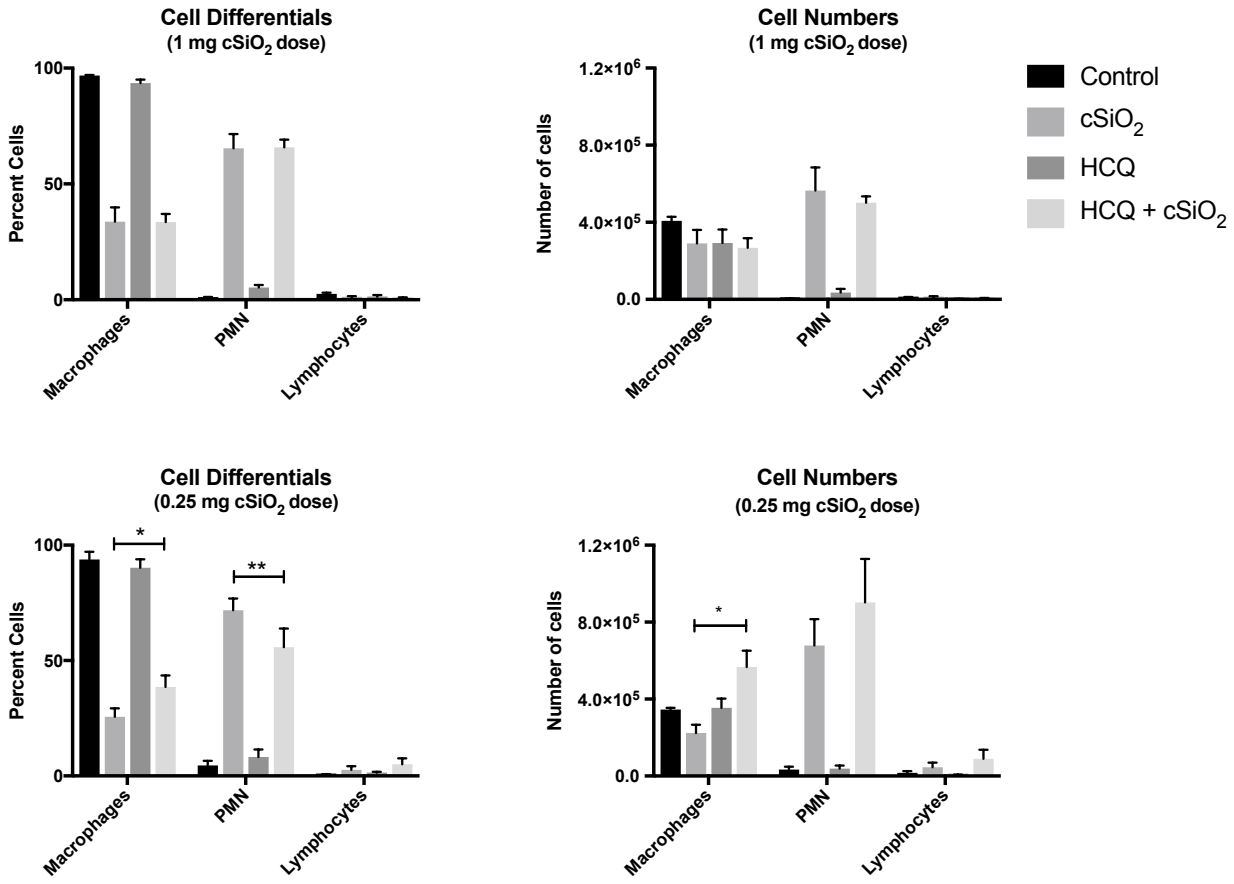

Figure 10: HCQ-treated mice retain more AM and have less infiltration of neutrophils.

Differential cell counts for 0.25 mg and 1 mg $cSiO_2$ treated mice. At 1 mg of $cSiO_2$, there were no differences in the percentage of cells or the raw cell numbers between the HCQ-treated mice and control mice ± $cSiO_2$ exposure. Mice exposed to $cSiO_2$ showed neutrophilic infiltration. At the 0.25 mg $cSiO_2$ dose, HCQ + $cSiO_2$ mice had a significant increase in the percentage of macrophages (*$p < 0.05$) and a significant decrease in the percentage of neutrophils (**$p < 0.01$) as compared to $cSiO_2$-exposed mice with no HCQ treatment. HCQ + $cSiO_2$ mice had significantly more macrophages in their lungs 24 hours after $cSiO_2$ exposure than did $cSiO_2$-exposed mice without HCQ treatment (*$p < 0.05$). $n = 4$-5

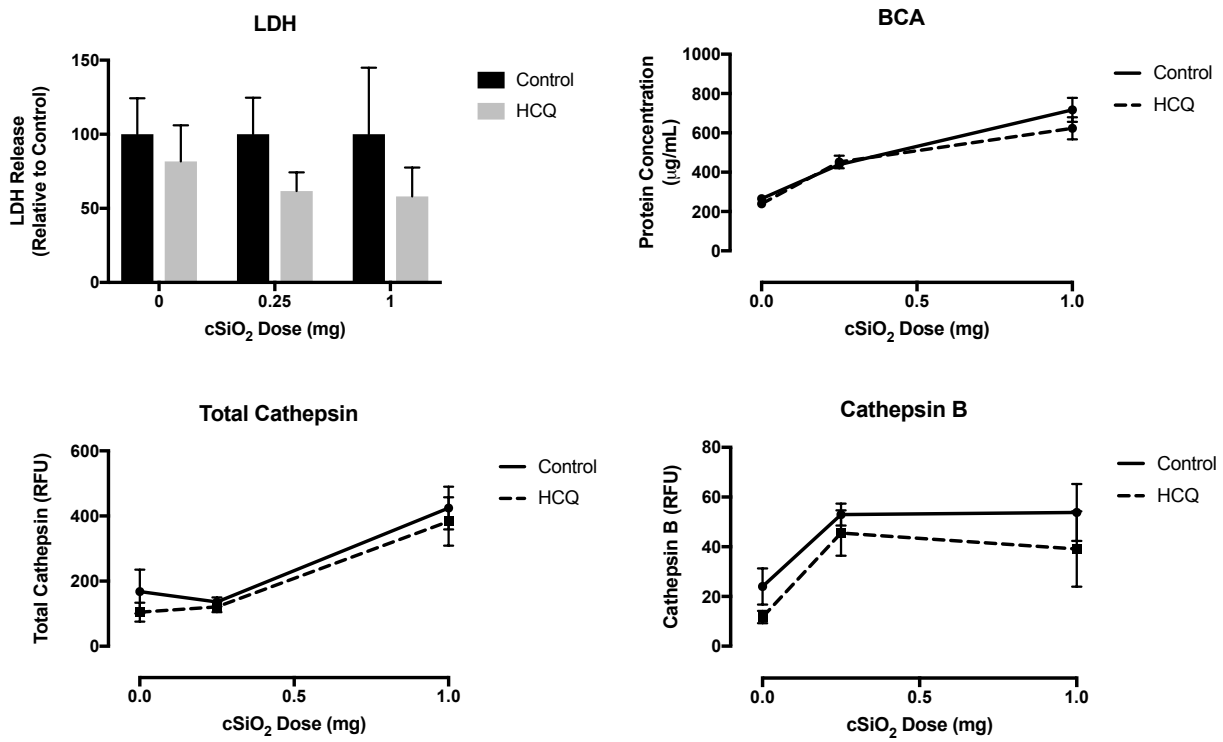

Figure 11: HCQ reduces $cSiO_2$ toxicity *in vivo*

LDH, total cathepsin, and cathepsin B release were lower in HCQ-treated mice at both 0.25 mg and 1 mg $cSiO_2$ exposures. The BCA assay showed a slight decrease in the amount of protein in the lung lavage fluid at the 1 mg $cSiO_2$ dose in HCQ-treated animals versus control. As expected the protein level increased as the $cSiO_2$ dose increased in both control and HCQ-treated mice. n = 4-5.

Table 1: Total Area Under the Curve for LDH Release

Total Area Under the Curve for LDH Release		
	Control	**HCQ Treated**
Total Area	241.3 ± 100.6	165.1 ± 52.58

3.6 *In vivo* cytokine production after HCQ treatment.

Cytokines from lung lavage supernatants were measured by a multiplex immunoassay (MSD U-PLEX, Meso Scale Diagnostics) because of the sensitivity and small sample size used (Figs. 12 and 13). Seven cytokines IFN-γ, IL-1β, IL-6, IL-10, IL-13, IL-33, and TNF-α were included in the 7-plex plate used for this experiment. IL-10 and IL-13 levels were below detection levels for both 0.25 mg and 1 mg $cSiO_2$-exposures and were not graphed. For mice exposed to 1 mg of $cSiO_2$, IFN-γ was only detectable in the $cSiO_2$ exposed group suggesting that HCQ was able to reduce IFN-γ after $cSiO_2$ exposure ($*p < 0.05$). While not significant, there was a trend that IL-6, IL-33, and TNF-α appeared to be lower with HCQ treatment in the 1 mg $cSiO_2$ exposed mice. IL-1β did not appear to be blocked with HCQ treatment. For the 0.25 mg dose, IFN-γ again appeared to be decreased by HCQ treatment, although in this case it was not statistically significant due to a large variance in the $cSiO_2$-exposed group. As in the 1 mg dose group, there is was a trend that HCQ + $cSiO_2$ mice seem to have lower levels of TNF-α as compared to $cSiO_2$ only mice. In all graphs, no visible bar signifies that levels were below detection.

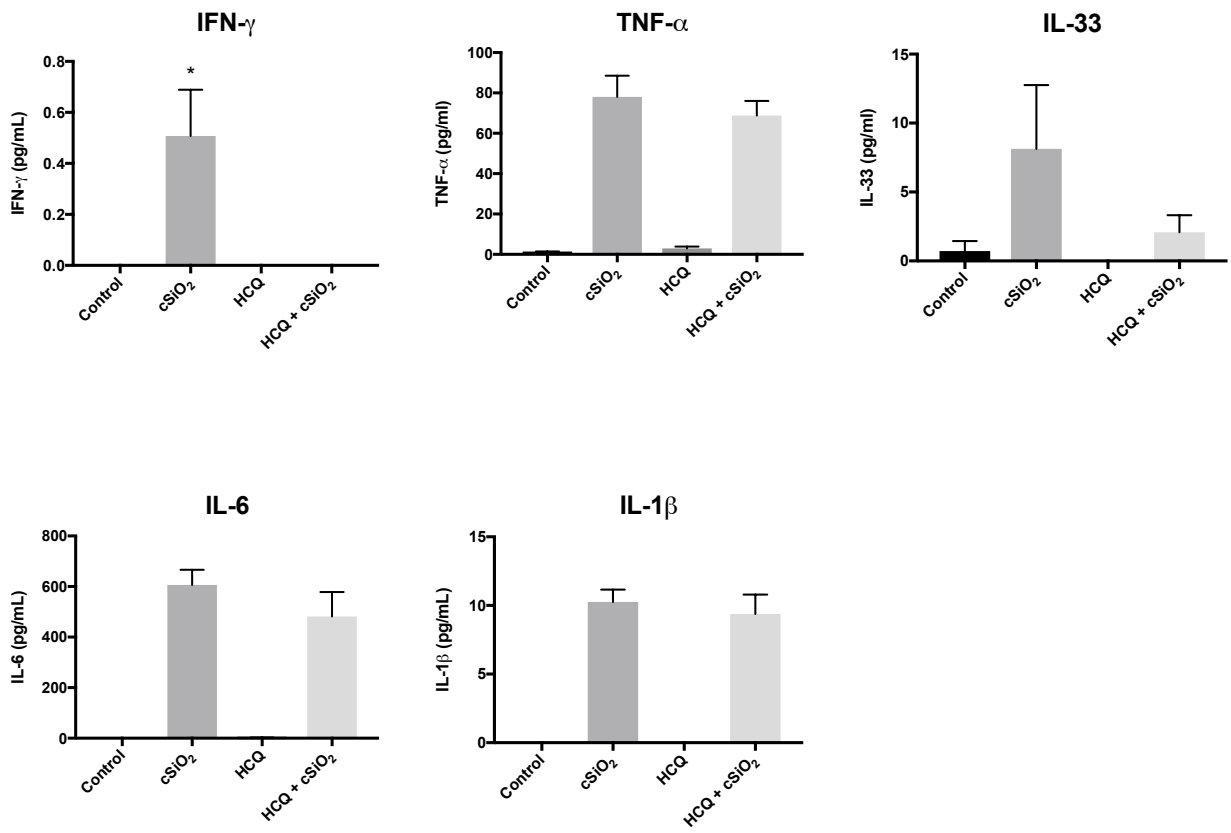

Figure 12: Cytokines after 1 mg SiO_2 exposure

Cytokines from lung lavage supernatants measured by a multiplex immunoassay. IFN-γ was only detectable in the $cSiO_2$-exposed group suggesting that HCQ was able to block IFN-γ after $cSiO_2$ exposure (*$p < 0.05$). While not significant, the data suggest a trend toward HCQ decreasing production of IL-6, IL-33, and TNF-α. IL-1β did not appear to be blocked with HCQ treatment.

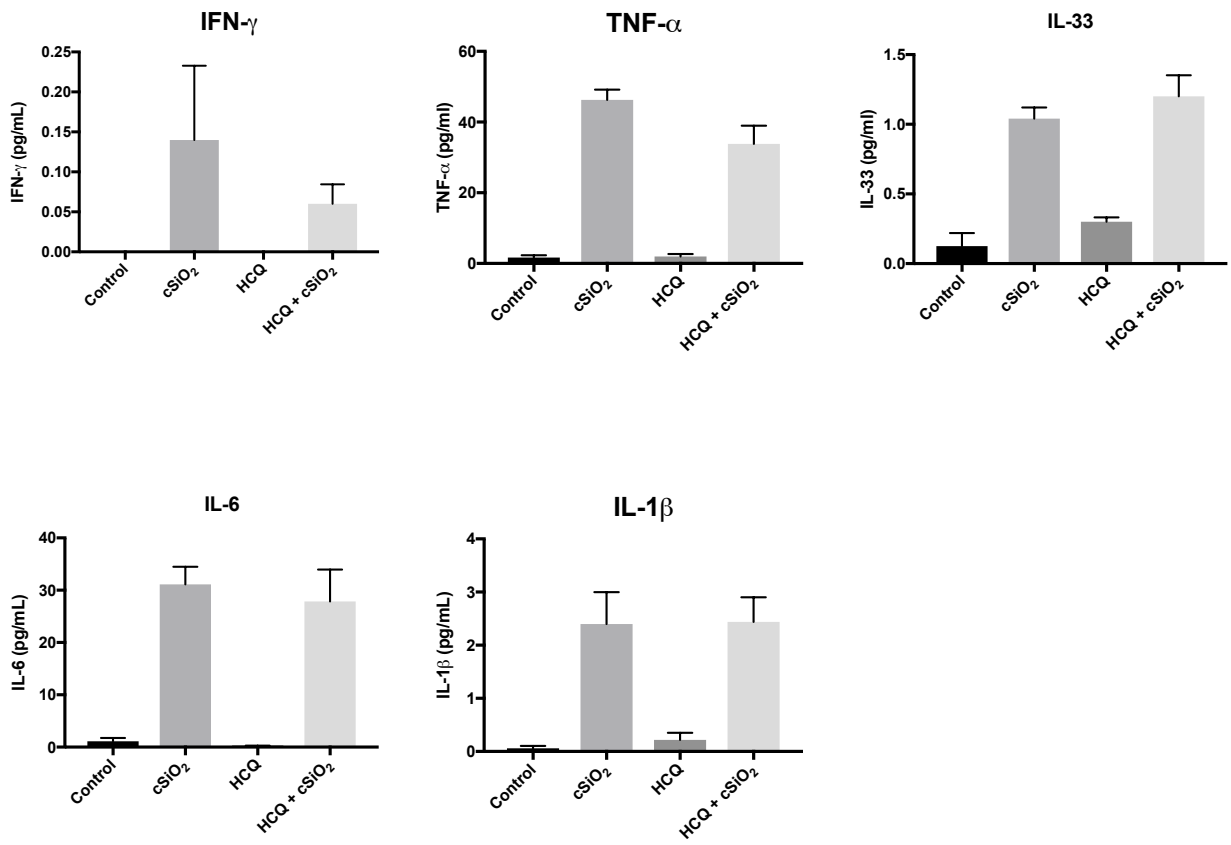

Figure 13: Cytokines after 0.25 mg SiO_2 exposure

For the 0.25 mg dose, IFN-γ appeared to be decreased by HCQ treatment, though not statistically significant due to a large variance in the $cSiO_2$-exposed group. As in the 1 mg dose group, there is a trend that HCQ + $cSiO_2$ mice seem to have lower levels of TNF-α as compared to $cSiO_2$ only mice. n = 4-5

3.7 HCQ blocks IL-1β production in *ex vivo* culture

Since HCQ was effective in blocking silica-induced IL-1β release from AM and the data showed that HCQ was taken up by lysosomes in AM, the AM from the mice were evaluated *ex vivo*. Cells collected during the whole lung lavage of mice treated with 0.25 mg of $cSiO_2$ *in vivo* were plated for *ex vivo* IL-1β culture and primed with LPS. After incubation, supernatants were assayed for IL-1β by ELISA. Data were normalized to IL-1β release per $1x10^5$ AM using the total cell count and percent of macrophages. Control and HCQ treated cells had very little IL-1β production as expected. Consistent with the *in vitro* data, cells from HCQ + $cSiO_2$ treated mice had a significantly less IL-1β production when compared to $cSiO_2$ exposed mice not treated with HCQ. These data are consistent with HCQ being able to prevent LMP after $cSiO_2$ exposure *in vivo*.

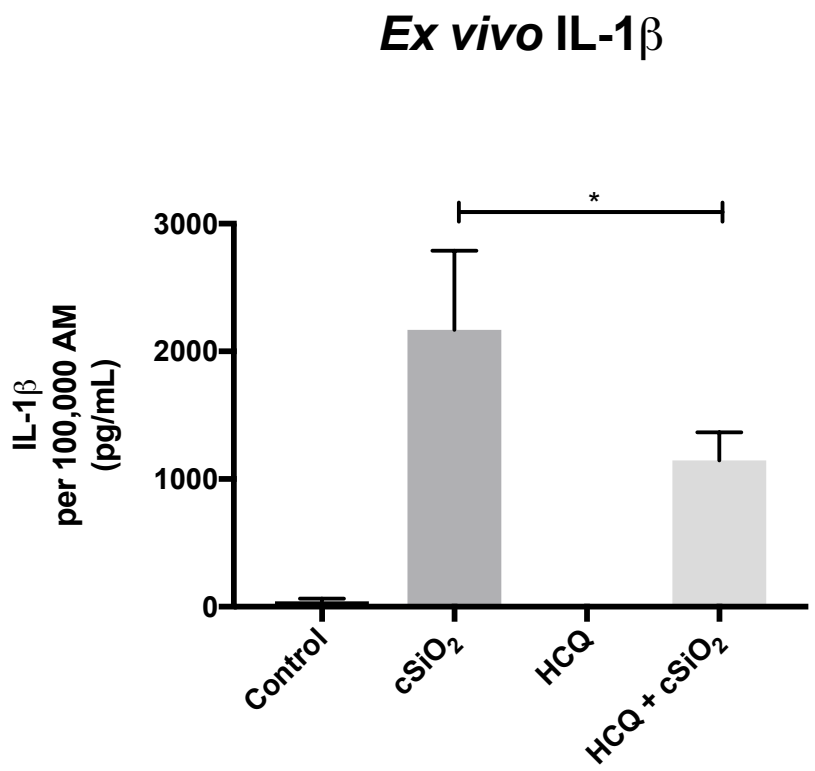

Figure 14: Ex Vivo Culture of C57Bl/6 AM

Cells collected during the whole lung lavage of mice treated with 0.25 mg of $cSiO_2$ *in vivo* were plated for *ex vivo* IL-1β culture. Data were normalized to IL-1β release per $1x10^5$ AM using the total cell count and percent of macrophages. Control and HCQ-treated cells produced only trace amounts of IL-1β. Cells from HCQ + $cSiO_2$ treated mice had significantly less IL-1β production when compared to cells from mice only exposed to $cSiO_2$ (*$p < 0.05$). These data are consistent with HCQ being able to prevent LMP after $cSiO_2$ exposure *in vivo*. n = 4-6

Chapter 4: Discussion

Silicosis is a progressive, fibrotic pulmonary disease that occurs after exposure to inhaled $cSiO_2$. The loss of pulmonary function resulting from silicosis causes significant morbidity and, in severe cases, mortality. Treatment options are limited to supporting respiratory function through supplemental oxygen and decreasing inflammation with corticosteroids (Leung et al., 2012). A growing body of evidence suggests that a key step in $cSiO_2$-induced lung inflammation is activation of the NLRP3 inflammasome resulting in the release of pleiotropic cytokines such as IL-1β and IL-18. At this time there is limited information on possible pharmaceutical approaches to regulation of NLRP3 inflammasome activity. However, it may be possible to regulate LMP, which has been proposed to precede NLRP3 inflammasome activation (Sayan & Mossman, 2016; Serrano-Puebla & Boya, 2016). When $cSiO_2$ is phagocytized by resident AM in an attempt to clear the particles from the lungs, the $cSiO_2$ compromises lysosomal membranes allowing lysosomal proteases to leak into the cytosol and induce NLRP3 inflammasome assembly (P Boya & Kroemer, 2008; Bunderson-Schelvan et al., 2016; Jessop et al., 2017). Therefore, blocking LMP would attenuate NLRP3 inflammasome assembly and could be a key mechanism to target in order to treat $cSiO_2$-induced inflammation. HCQ was chosen for this research because it is cationic amphiphilic drug (CAD) that is known to accumulate in lysosomes (Ben-Zvi et al., 2012; Patricia Boya et al., 2003). CADs diffuse through membranes in an unprotonated state but become protonated in the acidic environment of lysosomes, losing their ability to diffuse back out through lipid membranes. HCQ is used extensively as an anti-malarial and also to treat autoimmune diseases such as systemic lupus erythematosus (SLE). However, the precise mechanism to explain its action in SLE is unclear (Ben-Zvi et al., 2012).

The *in vitro* work from this study strongly supports the hypothesis that HCQ is able to block $cSiO_2$-induced LMP. HCQ was able to rescue BMDM from cell death after exposure to 50 or 100 μg/mL of $cSiO_2$ (Fig. 3). HCQ also caused significant reduction in the production of IL-1β in BMDM after $cSiO_2$ exposure (Fig. 4). These data are consistent with a 2017 paper by Chen *et al*. where they found that chloroquine (which differs from HCQ by one hydroxyl group) suppresses NLRP3 inflammasome activation in a murine model of endotoxic shock (X. Chen et al., 2017). Chen *et al*. showed that chloroquine is able to decrease IL-1β and IL-18 release from BMDM stimulated with LPS. They also found that chloroquine inhibits transcription of *Nlrp3* genes. Their research, however, did not examine the effect of HCQ on $cSiO_2$-induced toxicity.

Our research also directly measured the ability of HCQ to prevent LMP by measuring the release of β-*N*-acetyl-glucosaminidase (NAG) into the cytosol. HCQ was able to block NAG release in BMDM exposed to $cSiO_2$ at both 4 and 24 hours (Fig. 5). This data conflicts with data published by Boya *et al.* in 2003 where they argue that HCQ induces LMP. When cells were stained by immunofluorescence for cathepsin B, they saw that HCQ caused cathepsin B staining to change from being contained in lysosomes to being diffuse throughout the cell. They also concluded that HCQ causes mitochondrial membrane permeabilization and apoptosis. Boya *et al.* conducted their research in HeLa cells and also used some concentrations of HCQ which were higher than the concentrations used in this study (up to 60 μg/ml). They also did not measure LMP through a digitonin lysosomal permeability assay measuring NAG, but instead used microscopy to determine if LMP was occurring; therefore, it is hard to make direct comparisons between their research and the research presented in this paper (Patricia Boya et al., 2003).

The above *in vitro* results were predicated on the assumption that HCQ was being taken up into lysosomes. In order to test whether HCQ was becoming sequestered into the lysosomes, BMDM and AM were treated with LysoTracker. HCQ treatment reduced the amount of LysoTracker in lysosomes of both BMDM *in vitro* and AM *in vivo*. These results are consistent with HCQ sequestration into the lysosomes of these cells. The most likely mechanism by which HCQ is preventing the uptake of LysoTracker is due to HCQ increasing the pH of the lysosomes. Additionally, flow cytometry side scatter data from *in vitro* and *in vivo* experiments also confirmed that HCQ was not affecting the ability of cells to phagocytize the $cSiO_2$ particles; therefore, the toxicity results were not due to changes in cell interactions with particles.

Mice treated with HCQ *in vivo* showed modest reductions in $cSiO_2$-induced toxicity. Overall, LDH, total cathepsins, cathepsin B, IFN-γ and TNF-α trended lower in HCQ treated mice (Fig. 11-13). It was surprising that $cSiO_2$-induced increase in IL-1β in lung lavage fluid was not blocked *in vivo* by HCQ treatment; however, *ex vivo* culture of the AM from these same studies did result in significantly less IL-1β release in mice treated with HCQ. Several factors could have been responsible for the fact that there was not as pronounced of an effect *in vivo* as there was *in vitro*. It is possible that the dosing strategy and exposure time were not sufficient. Additionally, twenty-four hours may be too short of a time to see differences in the immune response. While these results could indicate that the dose of HCQ was simply too low, the *ex vivo* results suggest that there was enough HCQ sequestered into the lysosomes to block inflammasome formation and IL-1β production. The results from the *ex vivo* experiment may be explained by two differences: 1) the AM were primed for inflammasome activation with LPS when they were plated and 2) they were incubated for 24 hours in culture after isolation following *in vivo* $cSiO_2$ exposure. LPS

stimulates the NF-κB pathway increasing formation of the pro-form of IL-1β and increases the expression of NLRP3 inflammasome proteins (Sayan & Mossman, 2016). Therefore, it could be proposed that HCQ blocked IL-1β release by acting on the NF-κB pathway as previously proposed (X. Chen et al., 2017). In their studies, they pre-treated BMDM with chloroquine before adding LPS and saw a reduction of phospho-NF-κB p65 protein levels in chloroquine-treated cells. However, in the *in vitro* experiments in this study LPS was added prior to HCQ treatment thereby allowing activation of the NF-κB pathway by LPS to proceed prior to any potential inhibition by HCQ. Since, there was still a reduction in IL-1β production, it supports the notion that the action of HCQ to block IL-1β is not at NF-κB but rather at blocking LMP.

Previous research from our laboratory has shown that the tricyclic antidepressant, imipramine, was able to decrease $cSiO_2$-induced toxicity *in vitro* and *in vivo* (Biswas et al., 2017). Because HCQ and IMP are both lysosomotropic drugs that sequester in lysosomes, we hypothesized that they would have a similar ability to protect lysosomes from $cSiO_2$-induced LMP. The imipramine study showed similar results as HCQ in the ability to prevent IL-1β production *in vitro* and in an *ex vivo* culture. IMP was also able to decrease IL-1β levels in the lavage fluid *in vivo* after a 24-hour $cSiO_2$ exposure, which was not seen with HCQ. In long-term exposure studies, IMP-treated mice had less lung pathology and hydroxyproline levels. It is unknown whether other CADs may have similar protective effects on lysosomal membrane stability after particle exposure and further work should be done to better understand the interactions between CAD and $cSiO_2$ exposure. To our knowledge, no other work has been done linking a pharmaceutical intervention with the ability to block $cSiO_2$-induced LMP.

Chapter 5: Conclusions and Further Directions

The results of this study support the hypothesis that HCQ can prevent lysosomal membrane permeability from exposure to $cSiO_2$. The *in vitro* data strongly suggests that HCQ attenuates activation of the NLRP3 inflammasome and is doing this, at least in part, by blocking LMP. Cell death after $cSiO_2$ exposure was decreased with HCQ treatment *in vitro*. HCQ was able to block the production of IL-1β and the release of NAG into the cytosol in $cSiO_2$-exposed BMDM indicating that LMP was being prevented. This was the first study to show that HCQ is able to block LMP. While the *in vivo* data is less definitive, it also indicates that HCQ may be protective against $cSiO_2$ toxicity *in vivo*. These studies were able to confirm that HCQ is accumulating in lysosomes of AM when mice are treated with HCQ by gavage for 7 days. Data also indicated that HCQ was not affecting AM's ability to phagocytize $cSiO_2$ particles. LDH, total cathepsins, and cathepsin B, IFN-γ, and TNF-α levels trended lower in HCQ-treated mice. Increasing the power of the study by including more mice would improve the reproducibility of the studies and clarify these differences. It was surprising that the increase in $cSiO_2$-induced increase of IL-1β in the lung lavage fluid was not blocked *in vivo* by HCQ treatment; however, *ex vivo* culture of the AM did result in significantly less IL-1β release in mice that were treated with HCQ.

This study was limited by the short time-frame for the *in vivo* studies. Mice in this study were sacrificed 24 hours after $cSiO_2$ exposure in order to assess the acute inflammatory response; however, this time frame is too short to observe histological changes in the lungs. Future studies would benefit from longer-term exposures to discern if HCQ is able to block the development of fibrosis and $cSiO_2$-induced pulmonary pathology. Additionally, the dosage and treatment schedules were determined based on previous literature; however, in order to see more profound

effects from HCQ, it could be beneficial to increase the dose or lengthen the treatment period (X. Chen et al., 2017; Gómez-Guzmán et al., 2014). Additionally, this study only assessed the ability of HCQ to block $cSiO_2$ toxicity by pre-treating cells or mice with HCQ before exposure to $cSiO_2$. While it is beneficial to have options for prophylactic treatment for individuals who know they will be exposed to $cSiO_2$, such as military personnel deployed to arid regions, most treatments for $cSiO_2$ inhalation occur after an exposure has occurred. Therefore, it would be important to conduct further studies that examine the ability of HCQ to block $cSiO_2$-induced lung injury after exposure.

A large number of workers are exposed to airborne $cSiO_2$ which can contribute to the development of pulmonary and systemic disease. It is increasingly being recognized that lysosomes are important modulators of disease; however, there are virtually no pharmaceuticals directed at a lysosomal mechanism of disease. Many pharmaceuticals become sequestered in the lysosome through an ion-trapping mechanism, yet this has been considered an undesirable side-effect of the drugs. The data in this study show HCQ can prevent LMP induced by $cSiO_2$ by accumulating in lysosomes. It is unknown if other CAD have similar effects on lysosomal membrane stability, and examining the ways that these drugs interact with lysosomal membranes may provide insight into their ability to be used as treatments for inhaled particle exposures. Further work should be done to elucidate the how pharmaceuticals affecting lysosomes may be used to treat diseases caused by inhaled particles.

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