Regulation of silica-induced pulmonary inflammation

Rupa Biswas

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Regulation of silica-induced pulmonary inflammation

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Abstract

Inhalation of crystalline silica for an extended period of time results in inflammation in the lungs, which plays a pivotal role in the development of silicosis, a progressive, inflammatory and fibrotic pulmonary disease. Inhaled silica particles are encountered by alveolar macrophages (AM) in the lungs. Previous studies have indicated the role of scavenger receptors in binding of environmental particles and also demonstrated that AM recognize and bind silica particles through class A scavenger receptors (SRA) expressed on their surface. MARCO (Macrophage Receptor with Collagenous Structure) is a SRA family receptor and the predominant receptor for recognition and binding of silica particles by alveolar macrophages (AM). Respirable silica particles are recognized by MARCO on AM and are internalized through phagocytosis by AM and subsequently contained in phagosomes. The lysosomes fuse with the phagosomes forming phagolysosomes and deliver the lysosomal enzymes for degradation of the particulates. However, silica cannot be degraded and induce lysosomal membrane permeabilization (LMP), by as yet unknown mechanism. LMP results in the release of lysosomal proteases, to the cytoplasm. This triggers the assembly of the NLRP3 (Nucleotide-binding domain, and Leucine-rich Repeat containing family, Pyrin domain containing) inflammasome assembly and further inflammation as a consequence.

This project has focused on determining the relationship between MARCO and NLRP3 inflammasome activity and consequent downstream inflammation. Silica increased NLRP3 inflammasome activation and release of the pro-inflammatory cytokine, IL-1β, to a greater extent in MARCO/- AM
compared to wild type (WT) AM. A similar increase in NLRP3 inflammasome activation by silica was observed using MARCO Ab to block MARCO receptors in WT AM. Cathepsin B Inhibition (CA-074-Me), attenuated pro-inflammatory cytokine release. WT AM treated with cholesterol trafficking modifier U18666A decreased silica-induced NLRP3 inflammasome activation and MARCO null AM had reduced ability to sequester cholesterol following silica exposure. Taken together, this study demonstrates the critical role played by lysosomal membrane permeabilization (LMP) in triggering silica-induced inflammation and the contribution of MARCO in normal cholesterol uptake in macrophages. Therefore, in absence of MARCO, macrophages are susceptible to greater inflammatory response following silica exposure due to increased LMP.

This project also determined the anti-inflammatory potential of imipramine to inhibit pulmonary inflammation following acute and chronic silica exposure. Anti-inflammatory potential of imipramine was evaluated in vitro in isolated AM from C57Bl/6 WT mice, and in acute and chronic in vivo silica exposure model. The in vitro results demonstrated that pretreatment with imipramine significantly attenuated cathepsin B activity and IL-1β release. In acute in vivo model, pretreatment with imipramine alleviated silica-induced inflammation, as was evident from the decrease in the chemokine KC and neutrophil infiltration. In the chronic in vivo exposure model used in the co-administration study, the protective effect of imipramine was validated by the histopathological analysis of lung tissues and the hydroxyproline quantification of silica-induced lung fibrosis. In the chronic exposure model used in the post-administration study, though imipramine treatment after four weeks of silica exposure attenuated the silica-induced lung injury, imipramine treatment after four weeks of silica exposure reduced the chronic pathological condition. These experiments establish that the pretreatment or concomitant treatment of imipramine was effective in alleviating silica-induced lung pathology, signifying that the early and acute phase of silica-induced
inflammation is a critical phase where potential therapeutics interventions could be effective. Taken together, this project elucidates the role of MARCO in silica-induced NLRP3 inflammasome activation and inflammation, and establishes the protective role of imipramine in silica-induced inflammation and disease progression.
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List of key abbreviations

SR: Scavenger receptors
MARCO: Macrophage receptor with collagenous structure
AM: Alveolar macrophages
LMP: Lysosomal membrane permeabilization
IMP: Imipramine
A-Smase: Acid Sphingomyelinase
PAMP: Pathogen Associated Molecular Patterns
ELISA: Enzyme-linked immunosorbent assay
APC: Antigen Presenting Cell
LPS: lipopolysaccharides
OVA: ovalbumin
PBS: phosphate buffered saline buffer
BSA: bovine serum albumin
I.P: Intraperitoneal
O.P: Oralpharangeal
PF: Pulmonary fibrosis
List of publications and manuscript


Chapter 1 - Introduction

Silicosis is an inflammatory and fibrogenic lung disease caused by the inhalation of silica particles for an extended period of time. Silicosis is a progressive, debilitating disease, occasionally leading to a fatal outcome. Silicosis is a form of pneumoconiosis and is also known as Pneumonoultramicroscopic-silicovolcanoconiosis. It is one of the most ancient occupational illnesses and still remains a prevalent health problem throughout the world (ALA, 2010). It is a preventable disease but there is no cure for silicosis (Thomas and Kelley 2010). Currently, treatment choices for silicosis are limited and accompanied by complex adverse effects. Therefore it is crucial to determine the mechanism of silica-induced inflammation and disease progression, based on which potential therapeutics could be developed.

Silica

Silicon (Si) is one of most abundant mineral found on the earth’s surface (Rosenman et al 2003). Much of the silicon and oxygen on the earth’s crust is present as the compound silicon dioxide or silica (Hamilton et al 2008). The chemical formula for silica is SiO₂. Silica forms a tetrahedral structure and under natural condition, each silicon atom is covalently bonded to four oxygen atoms to form a large macromolecular structure, and the fundamental unit of this structure is the silicon centered tetrahedron SiO₄⁻⁴. These fundamental units are connected through the oxygen atoms that are shared between the two neighboring tetrahedral. Silica does not exist as a discrete molecule, but exist as a giant covalent structure. There are two natural forms of silica: crystalline and amorphous silica. In crystalline silica the tetrahedral units are arranged in an orderly sequence, which results in a repeatable pattern, whereas in amorphous silica the tetrahedral units are randomly oriented and form an irregular structural arrangement. There are seven polymorphs of crystalline silica, depending upon the temperature of formation. The main three polymorphs
are tridymite, cristobalite, and quartz (Elcosh, 2013). Quartz is the most common form of crystalline silica. Silica is very hard and has a very high melting point (1,610 °C) and boiling point (2,230 °C), is insoluble in water, and does not conduct electricity. These properties result from the very strong covalent bonds that hold the silicon and oxygen atoms in the giant covalent structure.

![Figure 1. This is a representation of the tetrahedral silica complex. Adapted from UC Davis ChemWiki](image)

Silica is a mineral compound and it is the basic component of the soil, sand, most types of rocks like granite, slate and mineral ore like quartz (ALA, 2013). Human exposure to silica can be due to environmental or occupational reasons. There are certain occupations where there is a high risk of silica exposure such as mining, foundry work, tunneling, sand blasting, rock drilling, manufacturing, farming and construction. While non-occupational exposures could be from dust storms or from wind erosions. Travelling on or living close to unpaved roads, construction site, agricultural fields, or quarries. Recreational activities can lead to silica exposure, for example pottery, gardening, sculpting (ALA, 2013).
Crystalline silica is an essential component of materials that have abundant uses in industry and are a vital component in many things used every day. It is present in bricks, mortar, and glass windows used for houses. It can be found in the engines and windscreen of cars and is needed for construction of roads and other transportation infrastructures, extensive range of sports and leisure facilities. Silica is found in daily items like glass and pottery and is used in a vast array of industries; the main ones are being glass, ceramic, foundries, construction, and the chemical industry. The finest form of silica is used as a functional filler for paints, plastics, and rubber. Silica sand is used in water filtration and agriculture. Crystalline silica is also irreplaceable in a series of high-tech applications, for example in optical data transmission fibers and precision casting. It is also used in the metallurgical industry as the raw material for silicon metal and ferrosilicon production, as well as in oil extraction. Silica is present in hundreds of products used in day to day life.

Exposure to crystalline silica is associated with more serious health effects compared to those caused by exposure to amorphous silica. Exposure to amorphous silica has been shown to cause self-limited reversible pulmonary inflammation (Warheit et al., 1995; Wilson et al., 1979). Epidemiological studies also demonstrated that workers with long-term exposure to amorphous silica do not show evidence of silicosis or COPD (Choudat et al., 1990; Wilson et al., 1979). Thus, exposure to amorphous silica does not lead to progressive pulmonary disease. However, crystalline silica has been recognized as an occupational hazard. Exposure to crystalline silica is relatively more detrimental to human health because of its structure and has been classified as a human lung carcinogen (Thomas and Kelley 2010). Exposure to crystalline silica over an extended period of time results in progressive pulmonary inflammation, and fibrosis know as silicosis.
Silicosis

Silicosis is a lung disease characterized by progressive inflammation and fibrosis. It is a disabling disease, which affects quality of life and may lead to death. Silicosis is caused due to environmental or occupational exposure to crystalline silica for an extended period of time. It remains a prevalent health problem throughout the world, particularly in the developing countries (GOHNET 2007).

Classification of silicosis

Classification of silicosis is made according to the disease's severity, including the radiographic pattern, onset, and rapidity of progression (OSHA 3177 - 2002). Silicosis can be broadly classified into three types which include:

- **Chronic silicosis:** Chronic silicosis results from long-term exposure for 10 or more years to crystalline silica in relatively low concentrations. Symptoms usually appear 10–30 years after first exposure. This is the most common type of silicosis. Patients with this type of silicosis may not develop obvious signs or symptoms of the disease early on, but abnormalities may be detected by x-ray. Chronic cough and exertional dyspnea are common. Radiographically, chronic simple silicosis reveals small opacities (<10 mm in diameter), typically rounded, and predominating in the upper lobes of the lung. Enlarged hilar lymph nodes with peripheral calcification - eggshell calcification can be seen.

- **Accelerated silicosis:** Accelerated silicosis develops 5–10 years after first exposure to higher concentrations of crystalline silica. Symptoms and x-ray findings are similar to chronic simple silicosis, but occur earlier and tend to progress more rapidly and are accompanied by severe respiratory impairment.
• **Acute silicosis**: Acute silicosis develops in less than 5 years after exposure to extremely high concentrations of respirable crystalline silica. Symptoms of acute silicosis include more rapid onset of severe disabling shortness of breath, cough, weakness, and weight loss, low blood oxygen level, often leading to death. The x-ray usually reveals a diffuse alveolar filling with air bronchograms, described as a ground-glass appearance.

## Signs and Symptoms

The clinical symptoms of silicosis may not develop until a few to many years after silica exposure. The long lag period between exposure and symptoms appearance makes it difficult to diagnose. Silicosis is at times misdiagnosed as pulmonary edema, pneumonia, or tuberculosis. The presence of silicotic nodules and a history of occupational exposure to silica dust are necessary for a positive diagnosis of silicosis (Mossman and Churg, 1998). The earliest symptom of silicosis is shortness of breath. Signs and symptoms of silicosis includes: dyspnea, chronic dry cough, fatigue, loss of appetite and weight loss, chest pain, diminished lung and vital capacity, cyanosis and cor pulmonale are later symptoms. As the disease progresses, the silicotic nodules coalesce and form a continuous mass of fibrotic tissue, called progressive massive fibrosis (Arch Pathol Lab Med 1988).

Silicosis is often accompanied by further complications, and there is increased risk for lung infections and tuberculosis. There is also risk of developing progressive massive fibrosis where severe scarring and stiffening of the lungs occur, which makes it difficult to breathe. Progressive massive fibrosis can occur in either simple or accelerated silicosis, but is more common in the accelerated form. Rapid progression of the disease can lead to respiratory failure and lung cancer.
Pathophysiology of silicosis

Silicosis is characterized by chronic inflammation, scarring in the form of nodular lesion in the upper lobes of the lungs, fibroblast proliferation, and collagen deposition and resulting in pathological development of irreversible pulmonary fibrosis (Craighead et al., 1988; Green and Vallyathan, 1996). With its potential to cause progressive physical disability, silicosis continues to be a significant occupational health illness around the world.

![Diagram showing pathophysiology of silicosis](image)

**Figure 2. Schematic showing pathophysiology of silicosis following silica exposure**

Histomorphologically silicosis is characterized by thickening of the alveolar interstitium, formation of hyalinized nodules, and collagen deposition resulting in the formation of silicotic nodules in the lung parenchyma (Huaux, 2007). These nodules are comprised of collagenous tissue arranged in a concentric pattern, with crystalline silica particles sequestered in the middle of the nodule with macrophages and
fibroblasts present on the periphery (Arch Pathol Lab Med 1988). Eventually the nodules coalesce leading to destruction and scarring of normal lung tissue.

**Prevalence of silicosis**

Silicosis remains a prevalent health problem throughout the world. Prevalence of silicosis is higher in developing countries compared to developed countries. The lower prevalence of silicosis in the developed countries could be attributed to usage of effective protective gear, or to improved working conditions for the workers. The seriousness of the disease is reflected by the fatalities and disabling illnesses that continue to occur among the workers exposed to crystalline silica. According to Occupational Safety and Health Administration (OSHA) report 2003 approximately over 2 million U.S workers are potentially exposed to crystalline silica, out of which more than 100,000 are in high-risk jobs.

**Current treatment options for silicosis**

Currently treatment choices for silicosis are limited and accompanied by complex adverse effects (Thomas and Kelley 2010). Most treatments used are aimed at relieving the pain and discomfort due to the symptoms and treating accompanied complications of silicosis. Most treatment choices available do not reverse or inhibit disease progression. Patients are administered oxygen to help increase blood oxygenation and bronchodilators to aid breathing. The commonly used pharmacological agents in the treatment of silicosis, such as a combination of corticosteroids and immunomodulatory drugs, often result in unpleasant and severe side effects such as toxicity and bone marrow suppression. It is important to determine the mechanism of silica-induced inflammation and disease progression, based on which new therapeutic approaches and potential therapeutic agents could be identified.
**Particle exposure and respiratory system**

The human respiratory system includes the following regions: the nasal cavity, pharynx, larynx, trachea, bronchi and their smaller branches, and the lungs. The lungs are the essential respiratory organs and their principal function is to transport oxygen from the atmosphere into the bloodstream, and to release carbon dioxide from the bloodstream into the atmosphere. The air enters through the nostrils and passes the pharynx and larynx into the trachea. The trachea divides into two main bronchi that enter the lungs. The bronchi continue to divide within the lungs and give rise to bronchioles. Bronchioles continue to branch and terminate in alveolar sacs. The alveolar sacs are made up of clusters of alveoli. Alveoli are tightly wrapped in blood vessels, and it is in the alveoli where the gas exchange occurs. An adult human lung contains an estimated 300 million alveoli. The ratio of total capillary to total alveolar surface is slightly less than one. Capillaries, blood plasma, and transformed blood elements are separated from the air space by a thin layer of tissue formed by epithelial, interstitial, and endothelial components.

The lungs are constantly exposed to a variety of pathogens and particles. Considering the surface area of the lung and the volume of air inspired on a daily basis, it is remarkable that negligible inflammation is observed under normal physiological conditions. Several defense mechanisms exist throughout the respiratory tract aimed at keeping the mucosal surfaces free from particles deposited by inhalation. Large inhaled particles > 5.0 μm are trapped by the surfactant proteins and the mucous lining in the trachea and then propelled out of the lung by the continuous beating of ciliated cells lining the mucosal surface. In contrast, small particles including respirable crystalline silica < 2.5 μm are deposited in the alveolar regions of the lung and are believed to have fibrogenic potential (Hanspeter et al., 2007).
Particle deposition in the respiratory system

Every day the respiratory system is exposed to large amounts of air through respiration. The calculated amount of air mobilized by an adult is 15 to 20 m$^3$ per day. Due to this large amount of air volume, a considerable amount of inhaled particles could accumulate in the lungs despite their low concentrations in the environment. The principal mechanisms of inhaled particle deposition in the respiratory tract are impaction, sedimentation, interception, and diffusion (McClellan, 1999; Raabe, 1999).

- Impaction is the dominant mechanism of deposition of particles larger than 3 µm in aerodynamic diameter. It occurs when the particles follow their initial path because of inertia rather than following changes in direction or speed of the airflow, resulting in collision with the wall of the airway usually near a bifurcation.

- Sedimentation of particles occurs when the airflow is slow and particle deposition is influenced by gravity. As particles increase in aerodynamic diameter the sedimentation increases.

- Interception is a non-inertial incidental contact with the airway wall. It is more important for fibers (e.g. asbestos) because their length (not their diameter) increases the probability that the ends of the fiber will deposit by interception on the walls of the airway.

- Diffusion is responsible for the deposition of particles smaller than 0.5 µm in diameter. As particle size decreases, diffusion increases. Deposition by diffusion is highest in the alveoli where the airflow is very slow.

Particle deposition can occur in any portion of the respiratory system, but for the development of silicosis, the deposit has to occur in the distal regions, such as the terminal bronchioles and alveoli. Particle size is usually a critical factor that determines the region of the respiratory tract in which a particle is
deposited. Large particles of size greater than 5 µm mass median aerodynamic diameter (MMAD) are usually trapped in the respiratory tract in the nasopharyngeal region and large conducting airways such as the trachea and larger bronchi. The smaller particles of (0.2- 5 µm MMAD) are transported to smaller airways and the alveoli.

**Alveolar Cells in the alveolus**

Small particles deposited in the alveoli come in contact with the alveolar cells. There are three major alveolar cells or pneumocytes in the alveolus: Type I, Type II and Macrophages.

- **Type I cells** - Type I cells form the structure of an alveolar wall. Type I cells cover a large surface area, approximately 90% of the alveolar surface. These cells have attenuated cytoplasm and appear to be poor in organelles. Since they constitute a large surface area of the lung they are prone to damage by various particle exposures. They are responsible for gas exchange at the alveolus.

- **Type II cells** - Type II cells are cuboidal and have abundant perinuclear cytoplasm. In case of damage to Type I cells, Type II cells undergo mitotic division to replace the damaged Type I cells (Witschi 1997). Type II cells are responsible for the production and secretion of pulmonary surfactants. The surfactants are important in maintain the normal respiratory mechanism by reducing alveolar tension. This reduces the energy required to inflate the lungs and reduces the likelihood of alveolar collapse during expiration. The Type II cells can undergo mitotic division and replace damaged Type I cells which are susceptible to vast numbers of toxic insults. Type II cells also contain the phase I enzyme, cytochrome P450, and are metabolically competent.

- **Alveolar Macrophages (AM)** - AM are primarily phagocytic cells which are loosely attached to the alveolar sacs. They act as the first line defense against numerous environmental challenges involving
pathogens, as well as inhaled particles, and function as an important link between the innate and adaptive immune responses (Gordon, 2007). They have various receptors on their surface (examples include Toll like Receptors, Scavenger Receptors, Mannose Receptors) to help recognize pathogen associated molecular patterns (PAMP) on pathogenic microorganism. Macrophages also contain cytochrome P450 and are capable of metabolism.

**Origin, differentiation, distribution and activation of Macrophages**

When respirable silica particles enter the pulmonary alveolus, they are internalized by phagocytosis by the alveolar macrophages (AM). AM are primarily phagocytic cells that are responsible for clearance of silica particles via the mucociliary escalator and/or lymphatic systems. AM are important for the innate immune response and in the initiation of adaptive immune response. AM are potent producers of acute response mediators such as IL-1β, which is an indicator of inflammasome activation and inflammation and has been associated with fibrosis development.

Macrophages originate from circulating monocytes and monocytes originate in the bone marrow from a common myeloid progenitor that is shared with neutrophils and monocytes are then released into the peripheral blood, where they circulate for several days before entering tissues and replenishing the tissue macrophage populations. The circulating monocytes give rise to a variety of tissue-resident macrophages throughout the body, as well as to other cells such as dendritic cells and Osteoclasts, which are distinct, irreversibly differentiated sub lineages. Once distributed through the blood stream, monocytes constitutively enter all tissue compartments of the body. Macrophages are abundant in every tissue in the body and display marked heterogeneity in phenotypes specific to each tissue, possibly due to local interactions with other cell
types in the tissue (Gordon, 2003). Resident macrophage populations in different organs, such as kupffer cells in the liver, alveolar macrophages in the lungs, Langerhans cells in the skin, osteoclasts in the bone and microglia in the central nervous system, adapt to their local microenvironment. Macrophages have also been reported to be derived from local proliferation of monocytes (Gordon and Taylor 2005). In initial experiments the radiosensitive precursors of monocytes in mouse bone marrow were transiently depleted with \(^{89}\)Sr, resulting in the loss of peripheral blood monocytes. However that did not affect the number of alveolar macrophages, supporting the role of local proliferation (Sawyer et al., 1982). Tissue-resident macrophages undergo local activation in response to various inflammatory and immune stimuli, the enhanced recruitment of monocytes and precursors from bone-marrow pools results in the accumulation of tissue macrophages that have enhanced turnover and an altered phenotype (Gordon and Taylor 2005). Tissue macrophages can be classically activated to M1/Classic macrophage by IFN\(\gamma\) and TNF signaling. M2b/Type II macrophage is mediated by Fc\(\gamma\)R ligation and TLR signal (Gordon, 2003). Tissue macrophages can be alternatively mediated to M2a/Alternative macrophages by IL-4 or IL-13. Tissue macrophages can be deactivated to M2c macrophage by TGF\(\beta\), IL-10 or GC. IFN\(\gamma\) plays a central role in MHC-II induction, enhanced microbial resistance and pro-inflammatory cytokine production (Migliaccio et al., 2005). While IL-10 suppresses pro-inflammatory cytokines and markers of activation, IL-4 and IL-13 have closely overlapping functions and induces an alternative activation phenotype in macrophages consistent with increased antigen presenting cell function and humoral responses.

Resident pulmonary macrophage populations have been divided into two major compartments: alveolar and interstitial, each expressing unique phenotypic markers (Laskin et al., 2001; Migliaccio et al., 2005). Under normal physiological conditions, the immune cell population in the airway or alveolar spaces is comprised mainly of AM (80-90\%) (Bowden, 1976; Devlin et al., 1994). While the primary function of AM
is phagocytosis, it is also known to play an important role in regulating inflammatory responses (Bowden, 1987). Murine AM are classified into unique subpopulations by their activation pathway and resulting cytokine secretion (Mosser, 2003). To maintain immune homeostasis a fraction AM releases immunosuppressive factors such as IL-10, IL-4, IL-13 and TGFβ (Barbarin et al., 2004; Hancock et al., 1998). On the other hand, immunoenhancing AM release proinflammatory factors such as TNF-α, IL-1β and IL-6 in response to triggers such as crystalline silica. AM are known to function as antigen presenting cells (APCs) and hence are important for regulation of adaptive immunity. With regard to human AM, at least two distinct subpopulations have been reported, one being immunosuppressive (RFD+1/RFD+7) and another small population function as immune activators (RFD+1/RFD-7) (Spiteri et al., 1992b). Both subpopulations differ in receptor expression and functional activity (Spiteri et al., 1992b). The immunosuppressive RFD+1/RFD+7 are good phagocytic cells, but poor APCs and immunoenhancing RFD+1/ RFD-7 are poor phagocytic cells and good APCs (Spiteri et al., 1992a) and can stimulate a T cell response, which could lead to an increased inflammatory response (Spiteri and Poulter, 1991). Moreover, the RFD+1/RFD+7 AM can suppress the RFD+1/RFD-7 AM-stimulated immune response. Consequently, the inflammatory status of a subject is extremely sensitive to the fine balance between these two subpopulations. Therefore, given the importance of balance between AM subpopulations, any imbalance of the system affects disease etiology.

Another macrophage phenotype located in the connective tissue of the lungs is known as the interstitial macrophage (Bowden, 1976). With respect to morphology and functions, alveolar and interstitial macrophages (IM) have striking differences. Morphologically AM are described as large mature cells while IM are smaller and resemble peripheral blood monocytes (Bowden, 1987; Zwilling et al., 1982). AM are also reported to have higher phagocytic ability as compared with the IM. Furthermore AM were effective in producing cytokines such as TNF-α and IFN-γ while IM produced higher levels of IL-1β and IL-6 and show
increased MHC-II expression along with greater antigen presenting activity (Franke-Ullmann et al., 1996). The role of IM in particle-induced diseases is unclear. However, both AM and IM play important roles in crystalline silica-induced pulmonary fibrosis. AM are phagocytic cells and play a role in phagocytosis and clearance of crystalline silica along with their well-established role in the production of cytokines and fibrogenic factors (Lehnert et al., 1989). In contrast, IM are responsible for trapping the crystalline silica in the interstitium and stimulating interstitial lymphocytes and fibroblasts to mount an immune response and promote collagen deposition (Adamson et al., 1991).

**Scavenger Receptors and their role in silica uptake and silica-induced inflammation**

Scavenger receptors (SR) are a family of cell surface glycoproteins expressed on AM. AM express numerous pattern recognition receptors, such as Toll like receptors, C-type lectin, β2 integrin and SR on their surface to recognize and bind environmental pathogens and particles (Platt and Gordon, 2001). The SR were first discovered in 1979. The scavenger receptors recognize a number of ligands, such as gram positive and gram negative bacteria, lipopolysaccharide (LPS), lipotechoic acid, polynucleotides and apoptotic eukaryotic cells (Elomaa et al., 1995; Kunjathoor et al., 2002; van der Laan et al., 1999). Members of the SR family display broad ligand-binding properties, which overlap between members of the family.

SR are mainly expressed on macrophages and dendritic cells, and are also found on nonimmune cells like epithelial cells, endothelial cells and hepatocytes (Murphy et al., 2005). There are eight known classes of SR designated A through H. They are divided into eight different classes (A-H) based on structure and function. (Murphy et al., 2005).
Previous studies have demonstrated that AM recognize and bind silica particles through class A scavenger receptors SRA expressed on their surface (Shankala et al., 2002; Hamilton et al., 2006; Arredouani et al., 2006). SRA are expressed predominantly as membrane receptors on macrophages and dendritic cells, mast cells, epithelial cells and endothelial cells (Brown et al., 2007; Murphy et al., 2005). SRA play a significant role in host-defense and are able to recognize and bind to a wide variety of ligands including acetylated proteins, polyribonucleotides, polysaccharides, environmental particles. Examination of the SR ligands illustrates that they are polyanionic in nature, although many polyanions do not bind SRA (Platt and Gordon, 1998). Specificity of their binding is determined by the negative charge on the ligand, the ligand structure, the relative affinity between the SRA and a particular ligand, and the availability and expression of various SRA. SRA I and SRA II are expressed primarily on macrophages and have been associated with silica binding (Hamilton, et al 2008). The SR most associated with AM silica binding are SRA I, SRA II and Macrophage receptor with collagenous structure (MARCO). MARCO is the predominant receptor for silica binding.

MARCO is expressed mainly on AM, macrophages in the marginal zone of spleen and lymph nodes, and dendritic cells. MARCO functions primarily as a phagocytic receptor binding to a variety of microbial components and can also modulate inflammatory signaling by Toll-like receptors. Both SRA I/II and MARCO exhibit overlapping and extensive ligand recognition capacity (Sarrias et al., 2004). MARCO also binds a variety of ligands such as AcLDL, gram positive and gram negative bacteria, LPS, and TiO2 (Arredouani et al., 2006; Elomaa et al., 1998). SR-AI, SR-AII and MARCO are associated with silica binding, and MARCO is the predominant receptor for binding and uptake of unopsonized particles such as silica.
SRA and MARCO share a similar structure as transmembrane proteins with an N-terminus cytoplasmic domain in addition to an extracellular trimeric collagenous domain, ending with a cysteine-rich C-terminus (SRCR). SRA also has α-helical coiled-coil domain not present on MARCO, but MARCO has more collagen repeats. SRA II lacks the SRCR C-terminus.

Figure 3. Schematic of class A scavenger receptors responsible for silica binding. Adapted from reference Hamilton, et al., 2008.

Role of AM in initiating inflammation and fibrosis following crystalline silica exposure

AM are present at the site of particle deposition in the alveoli. AM are primarily phagocytic cells and they are an important part of the innate immune system. Silica particles are recognized via SR on the surface of AM and are phagocytosed by AM. AM are responsible for clearance of crystalline silica particles from the lungs by the mucociliary pathway or by translocation to the interstitium and lymphatic system. Successful clearance of particles from the alveoli by AM results in nominal release of pro-inflammatory
factors and minimal cell injury and inflammation (Brody et al., 1982). However, if this chain of events is interrupted, it can lead to prolonged interaction of crystalline silica with pulmonary epithelial cells and other immune cells resulting in increased cell injury and translocation of crystalline silica to the interstitium. This could be due to particle overload because of continued exposure to high amounts of crystalline silica which is not cleared from the lungs due to overwhelmed clearance mechanisms.

Multiple mechanisms are proposed to be involved in inflammation and fibrosis development following silica exposure. According to one mechanism, high concentrations of particles in the lungs lead to translocation of particle laden AM to extra pulmonary sites such as the lymph nodes and interstitial space (Oberdorster, 1995). Once the particles are in the interstitium, they cannot be easily removed and subsequently interact with fibroblasts and epithelial cells that are in close proximity (Adamson et al., 1992). Consequently, the crystalline silica is phagocytosed by interstitial macrophages and the activated interstitial macrophages secrete pro-inflammatory cytokines and growth factors, which stimulate fibroblasts. The stimulated fibroblasts and epithelial cells secrete fibrogenic growth factors and cytokines that contribute to development of a persistent fibrotic condition of the lung (Adamson et al., 1991). The uncleared crystalline silica, which migrates to the interstitium, may also interact with the resident AM for prolonged periods of time. Some of the resident AM get activated and secrete pro-inflammatory cytokines, while a fraction of AM undergo apoptosis due to pronounced cytotoxic effects of crystalline silica (Huaux, 2007; Iyer and Holian, 1997). Some studies have suggested that the immunosuppressive AM population specifically undergoes apoptosis (Iyer and Holian, 1997).

In another mechanism, crystalline silica-exposed AM secrete inflammatory factors as cytokines, proteases. Some studies have shown that following silica exposure, they are internalized by phagocytosis by
the AM. Silica particles are contained in vesicles called phagosomes. The lysosomes fuse with the phagosomes forming phagolysosomes and delivering the lysosomal enzymes for the degradation of the silica. However, silica permeabilizes the lysosome leading to the release of lysosomal proteases to the cytoplasm. Lysosomal protease release can lead to apoptosis or onset of inflammation by triggering inflammasome activation. Inflammasome activation leads to IL-1β release, which has been indicated in inflammation and fibrosis development.

Activated AM also interact with immune and non-immune cells and contribute to the process of recruiting neutrophils, dendritic cells and lymphocytes. Among the cytokines released by AM, TNF-α and IL-1β have been reported to play critical roles in the development of silicosis (Shi et al., 1998). Most compelling is the evidence demonstrating anti-TNF-α antibodies or soluble TNF receptors and IL-1β antagonists’ attenuate crystalline silica-induced murine pulmonary fibrosis (Piguet et al., 1990; Piguet and Vesin, 1994; Piguet et al., 1993). A variety of inflammatory and immune responses are stimulated by TNF-α and IL-1β, including lymphocyte proliferation, oxidative bursts and degranulation of inflammatory cells (Herseth et al., 2008; Shi et al., 1998). Furthermore, analysis of TNF-α and IL-1β levels in the lavage fluid from crystalline silica-treated mice demonstrated an immediate increase in levels that are thought to be major contributors in the later development of inflammation and fibrosis (Driscoll et al., 1995).

**Role of Lysosomes in particulate exposure**

When respirable particulates, of 2.5 microns or less, enter the pulmonary alveolus, they are internalized by phagocytosis by the alveolar macrophages (AM). The particulates are contained in vesicles called phagosomes. The lysosomes fuse with the phagosomes forming phagolysosomes and delivering the
lysosomal enzymes for the degradation of the particulates. Lysosomes are cytoplasmic single membrane-bound vesicles that contain various lysosomal enzymes (Appelqvist et al., 2013). The nascent lysosomal enzymes are formed in the rough endoplasmic reticulum (RER) and are transported to the Golgi apparatus by vesicles. Pro-lysosomal enzymes bind mannose-6-phosphate receptors and are translocated to acidic lysosomal compartments where the ligand dissociates and the receptors recycle back to the Golgi (Kornfeld 1986). The pro-lysosomal enzymes are cleaved to form functional lysosomal enzymes, which are hydrolytic in nature. The hydrolytic enzymes degrade proteins, nucleic acid, lipids and carbohydrates. Lysosome functions include breakdown and degradation of intra and extracellular debris, microorganisms, apoptotic cells, old organelles and recycling of nutrients (Luzio et al., 2007). The hydrolytic enzymes have optimal activities at acidic pH. The lysosome lumen has an acidic pH of 4.5, which is maintained by an ATP-dependent proton pump that pumps protons from the cytosol into the lysosome (Boya and Kroemer, 2008). The lysosomal membrane is protected from the acidic hydrolases by lysosome specific expression of membrane proteins such as LAMP-1 and LAMP-2 (lysosome associated membrane proteins) that are heavily glycosylated and hence resist digestion by the hydrolases (Eskelinen 2006). Many agents and molecules can induce lysosomal membrane permeabilization (LMP), for example ROS, sphingosine, Bcl-2 family proteins, photo damage, cobra venom toxin and silica (Guicciardi et al., 2004). When the lysosomal membrane is damaged, lysosomal enzymes are released in the cytoplasm, triggering the onset of apoptosis, necrosis or inflammation.

**Inflammasome and pro-inflammatory cytokine formation**

Recent advances in understanding the inflammasome pathway have explained the role of this pathway in inflammation and fibrosis (Moretti and Blander 2014). Particles phagocytized by AM are
confined to intracellular vesicles called phagosomes, which undergo a series of interactions with endosomes and lysosomes (Johansson et al., 2010). Certain particles, such as silica, permeabilize the lysosome leading to release of lysosomal enzymes, including cathepsin B, to the cytoplasm (Boya and Kroemer, 2008; Guicciardi et al., 2004). This triggers the assembly of the inflammasome- a multiprotein complex. The inflammasome is an important component of both the innate and adaptive immune systems (Drenth and Van der Meer, 2006). The innate immune system provides immediate defense against infections and triggers the T and B cells of the adaptive immune system. Initial studies have identified the NLRP (Nucleotide-binding domain, Leucine-Rich Repeat and Pyrin domain containing Protein) family of proteins as a critical component of the inflammasome (Schroda and Tschopp, 2010). The NALP family of proteins plays a crucial role in alerting the mammalian immune system to the presence of dangerous conditions and pathogens (Hoffman and Wanderer 2010).

NLRP is a member of the NOD – like receptor (NLR) family. Other family members of NLR are NAIP (NLR family, apoptosis inhibitory protein) and NLRC4 (NLR family, CARD domain containing 4). The NLR family members all have similar structures, with a ligand binding leucine–rich repeat domain at the carboxyl terminus and an intermediary NACHT domain for nucleotide binding and self-oligomerization (Pedra et al., 2009). NLR family members also contain an effector domain at the amino terminus consisting of either a pyrin domain (PYD), a Caspase- activation and recruitment domain (CARD) or a baculovirus inhibitor of apoptosis protein repeat (BIR) (Pedra et al., 2009). It is thought that the main function of the NLR is regulation of pro-inflammatory cytokines such as IL-1β and IL-18. Though diverse molecular entities including bacteria, viruses, and components of dying cells, immune activators and crystalline or aggregated materials can activate NLR protein (NLRP), the precise mechanism of how NLRs recognize their ligands is unclear.
Out of the several NLRP proteins, the NLRP3, or cryoprin, inflammasome has been the most extensively studied. Though signals and mechanisms leading to inflammasome activation are still poorly understood, it has been reported that NLRP3 activation is required for stimulating inflammasome assembly. Once assembled, the inflammasome activates caspase-1, which catalyzes cleavage of the inactive precursor molecules pro IL-1β and pro IL-18 to their active forms, IL-1β and IL-18 (Franchi et al., 2009). IL-1β and IL-18 can only be secreted out of the cell in their active form (Dinarello 2009). Though Caspase-1 is important for cleavage of pro IL-1β and pro IL-18, there is evidence that these proinflammatory cytokines can be cleaved by a non Caspase-1 mechanism as well. For example, proteinase-3 has also been reported to cleave pro-IL-1β and pro-IL-18 to generate mature forms of these cytokines in the absence of Caspase-1 (Dinarello 2009). This may help explain the wide variety of signals that are known to activate the inflammasome.

In addition to NLRP, the adaptor protein ASC (apoptosis-associated speck like protein containing a Caspase recruitment domain (CARD)) is an important component of the inflammasome complex. Adaptor protein ASC connects NLRP proteins to pro-caspase-1 leading to cleavage of the CARD domain of pro-caspase-1 (Hoffman and Wanderer 2010). It has been reported by several research groups that in the absence of NLRP3 or ASC in genetically deficient mice, there is no activation or maturation of IL-1β. In fact, the ASC protein is required for bleomycin-induced IL-1β production and inflammation. Therefore, NLRP3 and ASC are potential therapeutic targets for treating chronic inflammation and fibrosis.

The exact composition of an inflammasome complex depends on the activator, which initiates inflammasome assembly. For example, NLRP-1 can directly bind and activate caspase-1 and/or caspase-5
via its unique CARD domain without the help of the adaptor protein (ASC). Once the inflammasome complex has been assembled, the downstream signaling due to activation of proinflammatory cytokines can contribute to the progression of many inflammatory diseases, such as silicosis.

**Silica-induced inflammasome activation**

Particles phagocytized by AM are confined to intracellular vesicles called phagosomes, which undergo a series of interactions with endosomes and lysosomes (Appelqvist et al., 2013). Certain particles, such as silica, permeabilize the lysosome leading to release of lysosomal enzymes, including cathepsin B, to the cytoplasm (Cassel et al., 2009; Hornung et al., 2008). This triggers the assembly of the inflammasome-a multiprotein complex (Gasse et al., 2009). The inflammasome assembly consists of NALP protein, the adaptor protein ASC [Apoptosis-associated Speck like protein containing a Caspase recruitment domain (CARD)] and pro-Caspase-1 (Drenth and Van der Meer 2006; Pedra et al., 2009). Adaptor protein ASC connects a NALP protein to pro-Caspase-1 leading to cleavage of the CARD domain of pro-caspase-1 resulting in activation of Caspase-1. At this point another signal is required, which is triggered by endotoxins like LPS to activate the NF-κB pathway. NF-κB, a transcription factor, is responsible for formation of pro IL-1β and pro IL-18 (Hoffman and Wanderer 2010). Active Caspase-1 catalyzes cleavage of the inactive precursor molecules pro IL-1β and IL-18 to their active forms, IL-1β and IL-18 (Hoffman and Wanderer 2010). IL-1β and IL-18 have been associated with inflammation and it is evident from the literature that inflammation and fibrosis development are closely linked to these inflammasome cytokines, the maturation and release of these cytokines (Cassel et al., 2009; Hornung et al., 2008).
Lysosomal membrane permeabilization resulting from silica exposure

When respirable particulates of 2.5 microns or less deposit in the pulmonary alveolus, they are internalized through phagocytosis by alveolar macrophages (AM) and subsequently contained in phagosomes. The lysosomes fuse with the phagosomes forming phagolysosomes and deliver the lysosomal enzymes for degradation of the particulates. The lysosome lumen has an acidic pH of 4.5, which is maintained by an ATP-dependent proton pump (Boya and Kroemer 2008; Guicciardi et al., 2004). The lysosomal membrane is protected from the acidic hydrolases by lysosome specific expression of membrane proteins such as LAMP-1 and LAMP-2 (lysosome associated membrane proteins), which are heavily glycosylated and hence resist digestion by the hydrolases (Boya and Kroemer 2008; Guicciardi et al., 2004). However, a number of particulates including silica cannot be degraded and induce lysosomal membrane permeabilization (LMP), by an as yet unknown mechanism.

LMP results in the release of lysosomal proteases, including cathepsin B, to the cytoplasm (Appelqvist et al., 2013; Johansson et al., 2010). Cathepsin B release triggers the assembly of the NLRP3 inflammasome, which consequently results in activation of caspase-1. Another signal is required, which can be triggered by endotoxin (LPS), to activate the NF-κB pathway. NF-κB is responsible for formation of pro-IL-1β and pro-IL-18 (Franchi et al., 2009). Active caspase-1 cleaves pro-IL-1β and pro-IL-18 to their active and secretable forms, IL-1β and IL-18 respectively. IL-1β and IL-18 have been closely associated with inflammation and with development of fibrosis (Cassel et al., 2009; Hornung et al., 2008).

Since LMP plays an important role in inflammasome activation and onset of inflammation, LMP can be an important target for novel therapeutics for the treatment of inflammatory diseases linked to lysosomal
instability. Therefore, a drug or an agent that can stabilize the lysosome and prevent membrane permeabilization has a potential to attenuate downstream inflammation. One potential anti-inflammatory agent is imipramine (IMP), which has lysosomotropic properties and is a FDA approved tricyclic antidepressant drug.

**Imipramine**

Imipramine is mainly used in the treatment of major depression, enuresis and attention deficit hyperactivity disorder. It has also been evaluated for use in panic disorder. Imipramine, affects numerous neurotransmitter systems known to be involved in the etiology of depression, anxiety, ADHD, enuresis and numerous other mental and physical conditions. Imipramine is similar in structure to some muscle relaxants, and has a significant analgesic effect and, thus, is very useful in some pain conditions. Depression has been linked to lack of stimulation of recipient neurons at a synapse. Imipramine acts as a classical anti-depressant, acting as a strong reuptake inhibitor of neurotransmitters serotonin and norepinephrine in neurons. Imipramine binds the sodium-dependent serotonin and norepinephrine transporters, thus preventing or reducing the reuptake of the neurotransmitters by neurons. This increases the availability of norepinephrine and serotonin (5-HT) as central neurotransmitters by blocking their neuronal re-uptake. Therefore, there is an increase in the concentration and persistence of the neurotransmitters in the synaptic cleft leading to enhanced neuronal transmission. Imipramine has 65% bioavailability with oral dose and the plasma half-life is between 16-24 hours. There is a 2-5 day latency period to attain steady state plasma levels. Imipramine undergoes hepatic metabolism and renal excretion. Desipramine is the main metabolite of imipramine and is excreted in 1-2 weeks. Desipramine also exerts some anti-inflammatory activity.
Imipramine’s effect on the lysosomes

Amine-containing drugs represent a very important class of therapeutic agents. For decades it has been known that weakly basic compounds can be sequestered into acidic organelles such as lysosomes. These compounds are often referred as lysosomotropic amines. Imipramine and its active metabolite Desipramine have structural and physiochemical similarities with know lysosomotropic drugs such as Chloroquin. Lysosomotropism is the process by which an agent enters the lysosome and accumulates in the lysosomal lumen. These compounds are lipophilic weak bases, which in deprotonated form passively diffuse the lysosomal membrane, get protonated inside the lysosome and increase the pH of the acidic environment of the lysosomal lumen. Therefore, the pH gradient between the cytoplasm and lysosome is reduced, resulting in the drug getting trapped inside the lysosome. This process is also known as pH partitioning or ion trapping. Lysosomotropism may be one of the possible mechanisms of imipramine uptake in the lysosomes. Imipramine uptake in the lysosome is important because it is anticipated that imipramine stabilizes the lysosome from the lysosomal lumen. Stabilization of the lysosome prevents triggering of the inflammasome
activation, and consequently downstream inflammation. Inside the lysosome, the lysosomotropic drugs may increase the lysosomal stability, thereby preventing the onset of inflammation by silica exposure. However, the exact mechanism of action by which imipramine stabilizes the lysosomal membrane is not clear. In addition, the role of imipramine in models of particulate-induced inflammation has not been examined. Therefore, the potential of imipramine to inhibit inflammation following silica exposure was examined in this study.
Specific Aims

The specific aims proposed in this project will test the central hypothesis that following exposure to silica, there is an escalation of lysosomal membrane permeabilization (LMP) leading to subsequent inflammasome activation in absence of MARCO receptor. Hence, it is anticipated that a lysosomotropic drug like imipramine (IMP) will stabilize the lysosomes, thereby preventing inflammasome activation and inhibiting downstream inflammation. In order to test this central hypothesis the following aims are proposed:

Specific Aim 1
The aim of this study is to determine the role of MARCO in inflammation subsequent to silica exposure.
Hypothesis: In the absence of MARCO receptor there is increased inflammasome activation and consequent downstream inflammation due to increased lysosomal membrane susceptibility following silica exposure.
For this study, AM isolated from C57BL/6 wild-type (WT) and MARCO null mice were used to determine the role of MARCO in silica-induced inflammasome activation and inflammation.

Specific Aim 2
To determine the anti-inflammatory effect of imipramine on silica-induced inflammation in short-term exposure study.
Hypothesis: Imipramine with lysosomotropic characteristic will stabilize lysosomes, thereby decreasing NLRP3 inflammasome activation and inhibiting downstream inflammation caused by exposure to silica.
In vitro studies were conducted to demonstrate that imipramine affected LMP and decreased NLRP3 inflammasome activation. In addition, in vivo studies were conducted to determine if imipramine could decrease silica-induced inflammation.
Specific Aim 3

To determine the effect of imipramine as a protective and a therapeutic agent in the long-term crystalline silica exposure model.

Hypothesis: Imipramine exhibits anti-inflammatory effect in the long-term silica exposed inflammation.

A co-administration study was done to determine the protective effect of imipramine. This is a 6 weeks or 42 days study. Mice were exposed to silica or vehicle by oropharyngeal aspiration once a week for 4 weeks and simultaneously treated with imipramine or sham. Imipramine and sham treatment continued for another 14 days after the exposure to silica or vehicle was discontinued. Osmotic pumps were subcutaneously implanted in the mice used to deliver the drug or sham for length of the experiment.

Post-exposure study was done to determine the therapeutic effect of imipramine after 4 weeks of silica exposure. Mice were given silica or vehicle by oropharyngeal aspiration once a week for 4 weeks. Mice were then treated with imipramine or sham for 6 weeks. Osmotic pumps were used for drug or sham delivery for 6 weeks.
References in Chapter 1


69. Matsumoto, A., Naito, M., Itakura, H., Ikemoto, S., Asaoka, H., Hayakawa, I., Kanamori,


80. Palecanda, A., Paulauskis, J., Al-Mutairi, E., Imrich, A., Qin, G., Suzuki, H., Kodama, T.,


Chapter 2 - Potential role of the inflammasome derived inflammatory cytokines in pulmonary fibrosis

In this review the role of the inflammasome and inflammasome-derived inflammatory cytokines in pulmonary fibrosis was explored. Inflammasome activation has been implicated to be in various diseases, such as rheumatoid arthritis, Muckle-wells syndrome. This project has broad ramification for the treatment of particle-induced lung disease, and for diseases due to inflammasome activation and lysosomal instability. Since there is some evidence that the IL-1β is involved in inflammation and fibrosis development this review evaluated the significance of inflammasome activation and pro-inflammatory cytokines on pulmonary fibrosis.

Abstract

Pulmonary fibrosis is a progressive, disabling disease with mortality rates that appear to be increasing in the western population, including the United States. There are over 140 known causes of pulmonary fibrosis as well as many unknown causes. Treatment options for this disease are limited due to poor understanding of the molecular mechanisms of the disease progression. However, recent progress in inflammasome research has greatly contributed to our understanding of its role in inflammation and fibrosis development. The inflammasome is a multiprotein complex that is an important component of both the innate and adaptive immune systems. Activation of proinflammatory cytokines following inflammasome assembly, such as IL-1β and IL-18, has been associated with development of PF. In addition, components of the inflammasome complex itself, such as the adaptor protein ASC have been associated with PF development. Recent evidence suggesting that the fibrotic process can be reversed via blockade of pathways associated with inflammasome activity may provide hope for future drug strategies. In this review we will
give an introduction to pulmonary fibrosis and its known causes. In addition, we will discuss the importance of the inflammasome to the development of pulmonary fibrosis as well as discuss potential future treatment options.

**Introduction**

Five million people worldwide are affected by pulmonary fibrosis (PF). According to the Pulmonary Fibrosis Foundation and Centre for Clinical Epidemiology and Biostatistics, there is an estimated 200,000 patients with PF of which more than 40,000 die each year in the United States alone [1]. The symptoms of PF are obscure and mimic other clinical pathologies such as chronic obstructive pulmonary disease, heart failure and aging, thus misdiagnosis can occur. As a consequence, the actual number of people affected by PF may be significantly higher than the reported figure. Furthermore, varying terminology and lack of standard diagnostic criteria have complicated collection of accurate data.

The average age at the time of diagnosis of PF is 60-65 years. However, in the United States mortality rates in younger populations are increasing. In recent years, the rate of diagnosis for PF has continued to rise in the older population and evidence suggests that mortality rates for both men and women with PF will continue to rise for the foreseeable future [2]. Many people with the disease live only three to five years after diagnosis, as there is no effective treatment for PF.

Most cases of PF are idiopathic in nature with unknown events initiating onset of the disease, although various risk factors have been identified. Importantly, PF is not a guaranteed outcome for every individual who is potentially at high risk—suggesting a multi-factorial mechanism is involved in
development of PF. One hypothesis suggests that key events in the development of PF include an acute inflammatory response following exposure to an injurious agent followed by abnormal tissue repair and disruption of the lung architecture [3]. Alternatively, it has been suggested that the idiopathic form of PF is largely an epithelial-dependent fibrotic process that functions independently of any inflammatory pathways [4]. This theory, along with a lack of efficacy for anti-inflammatory/immunosuppressive therapies in PF patients, has led to a re-evaluation of the role of inflammation in the development of PF [5]. Additionally, evidence in mice suggesting that fibrosis can develop with a minimal amount of inflammation has further complicated the issue [6]. Nevertheless, there is a large body of evidence implicating inflammatory pathways in the overall etiology of PF [7-9]. It is likely that studies exploring more specific arms of the immune system, such as regulation of the inflammasome, will shed new light on its role in the early development of PF.

In contrast to the idiopathic form of PF, there are many known causes such as exposure to respirable particles and toxicants, viral infections, oxidative stress and gastroesophageal reflux disease that have been shown to initiate an inflammatory response and may induce events associated with PF [10]. Once respired particles and/or pathogens arrive inside the alveoli, they are engulfed by alveolar macrophages, which play an important role in the recognition, uptake and clearance of particles from the lungs [11]. Normally, uptake of the particle by the macrophage results in phagosome formation. The phagosome can then fuse with the lysosome--releasing lysosomal enzymes and ultimately destroying the foreign matter [12,13]. However, in some cases, engulfing particles such as silica or asbestos causes lysosomal membrane permeabilization and release of cathepsins into the cytosol where they contribute to apoptosis signaling [14] and inflammasome activation [15,16]. At this stage, apoptosis results in incompletely digested particles remaining inside the lung for extended periods of time [17]. Unsuccessful clearance of these particles cause repetitive
inflammation due to prolonged interaction with both immune and non-immune cell populations. Furthermore, release of cathepsin B activates the inflammasome [18].

In addition to environmental exposures, approximately 3% of PF cases are due to adverse side effects of many prescribed drugs [19]. In fact, there are nearly three hundred drugs that have been associated with fibrotic lung diseases, a topic that is thoroughly reviewed by Camus et al [20]. Unfortunately, most cases of drug-induced PF occur during normal dosing regimens in a small percentage of susceptible individuals, making early detection and prevention difficult [20]. Drugs most commonly associated with irreversible cases of PF include amiodarone, bleomycin, non-steroidal anti-inflammatory drugs (NSAID), most alkylating agents [20] and methotrexate [21, 22]. Mechanisms of drug-induced PF are mostly unknown, however, evidence suggests that the inflammasome may play a role. For example, bleomycin has been used to induce PF in murine models in order to better understand molecular mechanisms [23]. It has been demonstrated that bleomycin-induced lung inflammation and remodeling is largely mediated by uric acid, which is released from dying cells upon injury or insult [24]. When uric acid is released from dying cells activation of the NALP3 inflammasome occurs, which results in enhanced IL-1β production and subsequent inflammation [24]. In addition, it is believed that bleomycin induces PF pathology through the IL-1R1/MyD88 signaling pathway [25]. Furthermore, a role for the NALP3 inflammasome in acetaminophen-induced hepatotoxicity was recently reported and may shed light on the mechanisms of NSAID-induced PF [26]. Lastly, as a folic acid analog, methotrexate is one of the oldest and most efficacious antineoplastic drugs. However, some studies have suggested that a link between the use of methotrexate and development of PF exists [21,22]. It is likely that the mechanism of methotrexate-induced PF involves modulation of an endogenous anti-inflammatory agent known as adenosine, which has been reported to have pro-fibrotic
effects in some experimental models [27] and reportedly activates the NALP3 inflammasome following release from necrotic cells [28].

In addition to complications caused by drug-induced PF, little progress has been made toward treatment of the disease. At this time, there is no pharmacological treatment known to restore normal lung tissue architecture, which is paramount to regaining normal lung function [29]. The treatment choices for PF are limited and are aimed at preventing more lung scarring, relieving symptoms and improving the quality of life [30]. Conventional treatment options for PF include antitussives, bronchodilators, corticosteroids, immunosuppressive/cytotoxic agents and antifibrotic agents [31-33] (see Figure 1). Drugs used to suppress the immune response have been used as a method for treating PF, but like other current therapies, these drugs have had little success in altering the normal course of PF disease progression [31, 34]. However, recent findings on the role of inflammatory pathways and the cytokines involved in those pathways may provide future direction for the development of new drugs for the treatment of PF. Traditionally, onset and progression of PF has been considered an irreversible and progressive disease. However, there is evidence to suggest that under certain conditions, the fibrotic process can be reversed—which has broad implications for PF patients.

Results in our lab demonstrate that collagen deposition, a key step in the fibrotic process, is reversible once exposure to asymmetric dimethyl arginine has been removed [35]. In addition, use of an interleukin-1 receptor (IL-1R) antagonist was able to both prevent and reverse bleomycin-induced pulmonary fibrosis in mice [36]. This suggests that drugs designed against components of the inflammasome may also have potential for future treatment regimens. The inflammasome is an important regulator of the proinflammatory cytokines, interleukin-1 beta (IL-1β) and interleukin-18 (IL-18). Both IL-1β and IL-18 have been linked to
PF development and pathogenesis while clinical and experimental studies point to a crucial role of IL-1β and IL-18 in acute and chronic inflammation [37]. Other evidence supporting the potential for reversal of PF pathology has been demonstrated through studies on IL-13, an inflammatory cytokine known to be involved in fibrosis [38-40]. This has significant implications for PF patients as it provides hope for future treatment strategies focusing on inflammasome activity.

Figure 1. Present treatment options for PF. Available treatment options for PF include antitussives and bronchodilators, which relieve symptoms of dry unproductive cough and bronchospasm, respectively. In addition, corticosteroids are often administered for sustained relief of bronchospasm. Immunosuppressive/cytotoxic agents are usually reserved for patients not responding to steroid treatment or suffering from adverse side effects. Lung transfer surgery is only considered in patients less than 65 years of age and can lead to fatal complications. Oxygen supplementation has positive outcomes on patient health. However, the personal inconvenience may affect patient compliance.
Causal factors in PF disease development

*Environmental Exposures:* While a high percentage of PF cases are idiopathic in nature, PF is also associated with environmental or occupational exposures to respirable asbestos particles, silica, and metal dust. Other common factors that can contribute to PF include a genetic predisposition, some medications and medical conditions. In addition, exposure to viral infections such as influenza A virus, hepatitis C virus, HIV virus and herpes virus -6 can also increase the risk for PF [10].

*Genetics:* PF has a complicated etiology that most likely contains a genetic component that is strongly influenced by environmental exposures. Several polymorphisms have been associated with development of PF. In addition, patients with accelerated clinical progression have different genetic profiles than less severe case studies [41]. Cases of familial PF follow an autosomal dominant pattern [42] while the pathological symptoms remain indistinguishable from non-familial cases with the exception of age of onset [43]. However, variable penetrance, even within genetically susceptible individuals and families, suggests that PF disease outcome is highly influenced by environmental factors [42].

Inflammasome activity

*What is the Inflammasome?*

The inflammasome is an important component of both the innate and adaptive immune systems [44]. The innate immune system provides immediate defense against infections and triggers the T and B cells of the adaptive immune system. Initial studies have identified the NALP (NACHT domain, Leucine-Rich Repeat and Pyrin domain containing Protein) family of proteins as a critical component of the inflammasome
The NALP family of proteins plays a crucial role in alerting the mammalian immune system to the presence of “danger” conditions and pathogens [46].

NALP is a member of the NOD – like receptor (NLR) family. Other family members of NLR are NAIP (NLR family, apoptosis inhibitory protein) and NLRC4 (NLR family, CARD domain containing 4). The NLR family members all have similar structures, with a ligand binding leucine –rich repeat domain at the carboxy terminus and an intermediary NACHT domain for nucleotide binding and self-oligomerization [47]. NLR family members also contain an effector domain at the amino terminus consisting of either a pyrin domain (PYD), a Caspase activation and recruitment domain (CARD) or a baculovirus inhibitor of apoptosis protein repeat (BIR) [48]. It is thought that the main function of the NLR is regulation of pro-inflammatory cytokines such as IL-1β and IL-18. Though diverse molecular entities including bacteria, viruses, and components of dying cells, immune activators and crystalline or aggregated materials can activate NLR protein (NLRP), the precise mechanism of how NLRs recognize their ligands is unclear.

Out of the several NALP proteins, the NALP3, or cryoprin, inflammasome has been the most extensively studied. Though signals and mechanisms leading to inflammasome activation are still poorly understood, it has been reported that NALP3 activation is required for stimulating inflammasome assembly [48]. Once assembled, the inflammasome activates caspase-1, which catalyzes cleavage of the inactive precursor molecules pro-IL-1β and pro-IL-18 to their active forms, IL-1β and IL-18 [49]. IL-1β and IL-18 can only be secreted out of the cell in their active form [50]. Though caspase-1 is important for cleavage of pro-IL-1β and proIL-18, there is evidence that these proinflammatory cytokines can be cleaved by a non Caspase -1 mechanism as well. For example, proteinase-3 has also been reported to cleave pro-IL-1β and
pro-IL-18 to generate mature forms of these cytokines in the absence of Caspase-1 [50]. This may help explain the wide variety of signals that are known to activate the inflammasome.

In addition to NALP, the adaptor protein ASC (apoptosis-associated speck like protein containing Caspase recruitment domain (CARD)) is an important component of the inflammasome complex. Adaptor protein ASC connects NALP proteins to pro-caspase-1 leading to cleavage of the CARD domain of pro-Caspase-1 [46]. It has been reported by several research groups that in the absence of NALP3 or ASC in genetically deficient mice, there is no activation or maturation of IL-1β [24,51-54]. In fact, the ASC protein is required for bleomycin-induced IL-1β production and inflammation [25]. Therefore, NALP3 and ASC are potential therapeutic targets for treating chronic inflammation and fibrosis.

The exact composition of an inflammasome complex depends on the activator, which initiates inflammasome assembly. For example, NALP-1 can directly bind and activate Caspase-1 and/or Caspase-5 via its unique CARD domain without the help of the adaptor protein (ASC) [55]. Once the inflammasome complex has been assembled, the downstream signaling due to activation of proinflammatory cytokines can contribute to the progression of many inflammatory diseases, including PF.

Inflammasome Activity and Fibrosis

Molecular mechanisms of acute lung injury resulting in inflammation and fibrosis are not completely understood. However, recent understanding of the inflammasome pathway has led to recognition for the role of the inflammasome in PF development and pathogenesis [49].
Some agents that have been associated with the development of the non-idiopathic forms of PF, such as asbestos and silica, have also been shown to induce lysosomal membrane permeabilization and assembly of the inflammasome [56, 57]. Permeabilization of the lysosomal membrane results in the release of proteases such as cathepsin B into the cytosol [53, 54]. Cathepsin B activates the NALP3 inflammasome and ultimately leads to IL-1β and IL-18 cleavage from their pro-isoforms [52, 58]. In addition, evidence suggests that maturation of IL-1β and IL-18 plays a critical role in acute and chronic inflammation, similar to what is observed in cases of PF [37]. For example, a role for IL-1β in fibrogenesis has been established using IL-1 receptor deficient mice, possibly through stimulation of the matrix metalloproteinases [59]. In fact, gene expression analysis of patients with histologically proven PF highlighted the importance of matrix metalloproteinase 7, an observation that was supported with studies using matrix metalloproteinase 7 knockout mice [60]. Similarly, neutralization of IL-18 has been shown to reduce obstruction-induced renal fibrosis [61] and is also known to induce gene expression of IL-1 [44]. IL-1β, in particular, is an important mediator of the inflammatory response following injury or infection [62] and may be a therapeutic target for chronic lung inflammation and fibrosis [25].

Characteristic changes in proinflammatory cytokine profiles associated with the inflammasome may also result in the recruitment of fibroblasts and inflammatory cells [63]. Fibroblasts and myofibroblasts provide a structural platform for the lung and may be key effectors in PF development. Activation of fibroblasts and myofibroblasts is a pathological hallmark of PF and most likely results from the downstream effects of abnormal cytokine, chemokine and growth factor activity. In areas with increased fibroblast activity, excessive deposition of extracellular matrix along with loss of the normal structural components of the lung is observed [64].
Downstream Effects of IL-1β and IL-18.

The proinflammatory cytokines of the IL-1 family, including IL-1β and IL-18, play important roles in antimicrobial host defense as well as modulation of gene expression. Once assembly of the inflammasome and subsequent activation of the cytokines IL-1β and IL-18 has occurred via cleavage of their pro-isoforms, a proinflammatory cascade of events is likely to follow. In vivo, IL-1β is primarily responsible for symptoms of acute inflammation such as fever, acute protein synthesis, anorexia, and sleep disturbances [65]. In contrast, IL-18 is largely involved in Th1 responses [44]. In addition, IL-1β and IL-18 play a primary role in diseases involving the innate and acquired immune systems such as PF, autoimmunity, rheumatoid arthritis, cancer, metabolic syndrome and atherosclerosis [66].

In renal fibrosis, activation of the inflammasome and subsequent release of IL-1β, along with TNF-α and IFN-λ, contributes to the epithelial mesenchymal transition [67], a critical step in disease development that may have implications for PF. Furthermore, long term exposure (>10 days) of human epithelioid dermal microvascular endothelial cells to IL-1β results in a permanent transformation into a myofibroblast phenotype [68], possibly increasing susceptibility for initiation of the fibrotic process. Further increases in IL-1β production will drive a subsequent increase in TGF-β, resulting in increased collagen production by fibroblasts—also known to be a critical step in the development of fibrosis [69]. Thus, IL-1β and IL-18 most likely contribute to favorable conditions for the development of fibrotic lesions such as increased collagen production and transformation of non-fibroblastic cells into a phenotype more typically seen in fibroblasts or myofibroblasts. It is clear that dysregulation of pathways leading to inflammation and the subsequent development of fibrosis is closely linked to the proinflammatory cytokines IL-1β and IL-18, which in turn are dependent on formation of an inflammasome complex for activation.
Current and future treatment options for PF

Current Treatment Options in Clinical Trials.

Current pharmacological treatment of PF is based on a combination of corticosteroids and immunosuppressants. However, the efficacy of treatment remains a matter of debate and these medications have serious adverse side effects. It is beyond the scope of this article to include a discussion of all current treatment regimens, however, there are several comprehensive reviews discussing the outcomes of trials aimed at testing standard therapies for PF [73, 74].

New Pharmacological Agents

In recent years there has been an increase in the launch of new pharmacological agents that are currently in clinical trial stage. Some of these therapeutic agents are interferon gamma, N-acetyl cysteine, etanercept (anti-tumor necrosis factorα), bosentan (anti-endothelin dual receptor antagonist) and imatinib (tyrosine-kinases inhibitor of the PDGF receptor) to name a few.

Agents involved in the inflammasome pathway are currently being studied as potential therapeutics for PF as well. For example, the successful use of the IL-1 receptor antagonist, anakinra, to treat bleomycin-induced PF in mice has raised the possibility for its use in human trials [36]. In fact, anakinra is currently being used for the treatment of other inflammatory diseases such as Muckle Wells Syndrome [75] and rheumatoid arthritis [76]. In addition, inhibition of cathepsin B--an inflammasome activator, has significant potential for therapeutic application in diseases linked to lysosomal dysfunction such as PF [54]. Specifically, Liu et al [77] reported a mechanism by which the transcription factor NF-κB can inhibit inflammation, cell death and the subsequent formation of fibrosis through its regulation of the serine protease
inhibitor 2A—a potent inhibitor of cathepsin B [78]. Therefore, it is possible that drugs aimed at other components involved in inflammasome activation and activity could have potential to be highly efficacious in reversing or lessening the initial effects associated with onset of PF (see Figure 2).

Conclusions

Pulmonary fibrosis is a progressive, disabling and fatal disease. Present treatment options for PF are inadequate due to an incomplete understanding of the molecular mechanism of the disease progression. Recent research focused on understanding the molecular mechanism of PF development has suggested that inflammasome activation may play a role in initiation and/or progression of the disease. In addition, inflammasome activation is involved in the development of other inflammatory and fibrotic diseases, which may provide clues for our current understanding of PF. Specifically, the NALP3 protein is most likely responsible for inflammasome activation leading to proIL-1β and proIL-18 cleavage and maturation by active caspase-1, which contributes to fibrosis development. In fact, evidence suggests that maturation of IL-1β and IL-18 plays a critical role in acute and chronic inflammation, similar to what is observed in cases of PF [37] and may result in the recruitment of fibroblasts and inflammatory cells [63]. Fibroblasts and myofibroblasts provide a structural platform for the lung and may be key effectors in PF development. Activation of fibroblasts and myofibroblasts is a pathological hallmark of PF and most likely results from the downstream effects of abnormal cytokine, chemokine and growth factor activity, such as what is observed following inflammasome activation.

In addition to aberrant regulation of cytokines, the adaptor protein ASC is an important component of the inflammasome complex and is known to play a crucial role in the development of bleomycin-induced PF
Therefore, identifying critical components of the inflammasome complex and its downstream effectors, such as NALP3, ASC, IL-1β and IL-18 may be key to the development of potential therapeutic targets, which will be important for novel PF treatments in the future.

Figure 2. Potential inflammasome targets for novel PF therapeutics. Lysosomal membrane permeabilization plays an important role in cathepsin B release and inflammasome activation. Therefore, stabilization of the lysosomal membrane would prevent initiation of the adverse events associated with inflammasome activation. Similarly, inhibition of cathepsin B would attenuate inflammasome assembly and activation. Further downstream of inflammasome assembly, inhibition of Caspase -1 would prevent maturation of proinflammatory cytokines such as IL-1β and IL-18. In addition, IL-1R antagonists would prevent the downstream events following the binding of IL-1β to its receptor.
References in Chapter 2


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70. Davis, M.J. and J.A. Swanson, *Technical advance: caspase-1 activation and IL-1{beta} release correlate with the degree of lysosome damage, as illustrated by a novel imaging method to quantify phagolysosome damage*. J Leukoc Biol, 2010.


Chapter 3 -Role of lysosomes in silica-induced inflammasome activation and inflammation in absence of MARCO

Abstract

Exposure to crystalline silica particles over an extended period of time results in pulmonary inflammation, which plays a vital role in pathological development of silicosis in humans. MARCO (Macrophage Receptor with Collagenous Structure) is the predominant receptor for recognition and binding of silica particles by alveolar macrophages (AM). Previously, it was shown that mice null for MARCO have a greater inflammatory response to silica, but the mechanism was not described. The aim of this study was to determine the relationship between MARCO and NLRP3 inflammasome activity. Silica increased NLRP3 inflammasome activation and release of the pro-inflammatory cytokine, IL-1β, to a greater extent in MARCO-/− AM compared to wild type (WT) AM. A similar increase in NLRP3 inflammasome activation by silica was observed using MARCO Ab to block MARCO receptors in WT AM. Furthermore, in MARCO-/− AM there was greater cathepsin B release from phagolysosomes, Caspase-1 activation and acid sphingomyelinase activity compared to WT AM, supporting the critical role played by lysosomal membrane permeabilization (LMP) in triggering silica-induced inflammation. The difference in sensitivity to LMP appears to be in cholesterol recycling since increasing cholesterol in AM by treatment with U18666A decreased silica-induced NLRP3 inflammasome activation, and cells lacking MARCO were less able to sequester cholesterol following silica treatment. Taken together, these results demonstrate that MARCO contributes to normal cholesterol uptake in macrophages, therefore, in the absence of MARCO, macrophages are more susceptible to a greater inflammatory response by particulates known to cause NLRP3 inflammasome activation and the effect is due to increased LMP.
Introduction

Silica is a basic component of soil, sand and most types of rocks. Silica forms a tetrahedral structure and there are two common forms of silica- crystalline silica and amorphous silica. Crystalline silica has a regular tetrahedral structure, while amorphous silica has an irregular structural arrangement. Environmental or occupational exposure to crystalline silica particles over an extended period of time results in pulmonary inflammation, which plays a vital role in pathological development of silicosis. Silicosis is a lung disease characterized by inflammation and fibrosis, and remains a prevalent health problem throughout the world. Currently, treatment choices for silicosis are limited and at present no cure exists for silicosis [1, 6]. Consequently, it is important to further define mechanisms of silica-induced inflammation on which new therapeutic approaches could be developed.

Inhaled silica particles are encountered by alveolar macrophages (AM) in the lungs. The AM are the primary innate immune phagocytic cells at the air tissue interface responsible for clearance of particles through the mucociliary escalator and/or lymphatic systems [6]. Previous studies have demonstrated that AM recognize and bind silica particles through class a scavenger receptors (SR) expressed on their surface [2, 3, 7, 21]. The class A SR family includes SR-A, and MARCO, which function primarily as phagocytic receptors binding to a variety of microbial components and can also modulate inflammatory signaling by Toll-like receptors [33, 34]. SR-AI, SR-AII and MARCO are associated with silica binding, and MARCO is the predominant receptor for binding and uptake of unopsonized particles such as silica [7, 8, 9, 10, 22, and 23].
Previous *in vivo* results showed an increased inflammatory response in MARCO<sup>−/−</sup> mice compared with WT mice following 24 hrs of silica exposure [26]. There was an increase in total protein levels, total number of lavage cells and a significant increase in infiltration of immune cells such as AM, DC and neutrophils in MARCO<sup>−/−</sup> mice compared with WT mice, all indicating an increase in inflammation in MARCO<sup>−/−</sup> mice. However, the mechanism to explain the increased inflammatory response in the absence of MARCO was not clear.

Recent advances in understanding the NLRP3 inflammasome have explained the role of this pathway in inflammation and fibrosis. Particles phagocytized by AM are confined to intracellular vesicles called phagosomes, which undergo a series of interactions with endosomes and lysosomes [4, 30, 31, and 32]. Certain particles, such as silica, permeabilize the lysosome leading to release of lysosomal enzymes, including cathepsin B, to the cytoplasm [27, 29]. This triggers the assembly of the NLRP3 inflammasome-a multiprotein complex [28]. The inflammasome assembly consists of NALP3 protein, the adaptor protein ASC [Apoptosis- associated Speck like protein containing a Caspase recruitment domain (CARD)] and pro-Caspase-1 [11, 12]. Adaptor protein ASC connects a NALP3 protein to pro-Caspase-1 leading to cleavage of the CARD domain of pro-Caspase-1 resulting in activation of Caspase-1. At this point another signal is required, which is triggered by endotoxins like LPS to activate the NF-κB pathway. NF-κB, a transcription factor, is responsible for formation of pro IL-1β and pro IL-18 [14]. Active Caspase-1 catalyzes cleavage of the inactive precursor molecules pro IL-1β and IL-18 to their active forms, IL-1β and IL-18 [14, 15]. IL-1β and IL-18 have been associated with inflammation and it is evident from the literature that inflammation and fibrosis development are closely linked to the maturation and release of these inflammasome cytokines [13, 16, 17, 18, 19].
We propose that the MARCO receptor can modulate the sphingomyelin pathway; leading to the lysosomal accumulation of sphingomyelin, phosphatidylcholine and cholesterol, and a decrease in ceramide. The sphingomyelin pathway is a ubiquitous signaling system, which activate multiple signal transduction pathways associated with both physiological and pathological processes that includes cell growth, cell death, autophagy, angiogenesis, cancer and inflammatory responses [40]. This pathway is initiated by the hydrolysis of membrane phospholipid sphingomyelin to ceramide. Increased ceramide production has been indicated in inflammation in response to a large variety of stressors [36]. It is anticipated that accumulation of cholesterol in the lysosomal membrane can contribute to lysosomal membrane stabilization and prevention of inflammasome activation and downstream inflammation [37, 38, and 39]. Furthermore, we propose that in the absence of MARCO receptor there is decreased cholesterol accumulation in the lysosomal membrane making them susceptible to membrane permeabilization upon exposure to silica. In this study we examined the effect of MARCO on inflammasome activation upon exposure to silica. For this study, AM isolated from C57BL/6 wild-type (WT) and MARCO null mice were used to determine the role of MARCO in silica-induced inflammasome activation and inflammation.
Results and Discussion

Evaluation of the effects of silica on IL-1β production in C57BL/6 WT and MARCO−/− AM

Since the molecular mechanisms involved in increased inflammation in MARCO−/− compared to WT mice are not completely understood, an in vitro study was conducted to measure release of key cytokines and activity of key enzymes in AM of WT and MARCO−/− mice. AM from MARCO−/− and WT mice were stimulated with LPS (20 ng/ml) to activate NF-κB and pro-IL-1β production, AM were then treated with 0, 25, 50, 100 or 200 µg/ml of silica for 24 hrs. Silica caused a dose-dependent increase in IL-1β release from MARCO−/− and WT AM. IL-1β release was significantly higher in MARCO−/− AM than WT AM at dosage values 100 µg/ml and 200 µg/ml (Figure 1 a). Maximum IL-1β was released at 100 µg/ml and decreased at 200 µg/ml in both MARCO−/− and WT AM, most likely due to cytotoxicity. The increased production of IL-1β by MARCO−/− AM relative to WT AM is consistent with the observation of increased inflammation in vivo in MARCO−/− mice [26].

In order to determine whether the increase in IL-1β release from MARCO−/− AM was due to the absence of MARCO receptor and not an unknown compensatory mechanism, MARCO Ab was used to block MARCO function in WT AM. IL-1β release from WT AM treated with LPS, silica and MARCO Ab was also significantly higher than WT AM treated with LPS and silica only (Figure 1b). Furthermore, the resulting levels of IL-1β release by blocking MARCO receptor function using MARCO Ab with WT AM were similar to levels of IL-1β release from MARCO−/− AM treated with LPS and silica. This result implies that the difference in IL-1β release between the WT AM and MARCO−/− AM can be attributed to the absence of MARCO function.
**Effect of silica on cathepsin B activation**

Lysosomal membrane permeabilization (LMP) leads to cathepsin B release from the lysosomal lumen to the cytoplasm [27, 29]. Release of cathepsin B to the cytosol triggers NLRP3 inflammasome assembly and subsequent downstream inflammation. In order to determine whether the effect of MARCO was at LMP, the contribution of silica on cathepsin B release to the cytosol was determined (Figure 2). Primary AM from MARCO−/− and WT mice were stimulated with or without LPS and then treated with silica (100 µg/ml) for 2 hrs prior to cathepsin B assay. Cathepsin B activation was higher in AM treated with silica alone or silica plus LPS treated AM from MARCO−/− mice compared to WT AM. The results indicate that silica exposure increases cathepsin B activity, and corresponds with increased lysosomal membrane permeabilization, which was increased in the absence of MARCO.

**Effect of MARCO on IL-1β**

The activation of NLRP3 inflammasome is known to be essential for maturation of IL-1β [18] and Cathepsin B has been proposed to work upstream of NLRP3 inflammasome assembly [41]. Therefore, the contribution of cathepsin B to silica-induced IL-1β release from AM was examined. As expected, AM pretreated with LPS and a cathepsin B inhibitor (10 µM CA-074-Me added 30 min prior to silica exposure) showed a significant reduction in IL-1β release compared to the absence of the cathepsin B inhibitor (Figure 3). The MARCO receptor is known to bind LPS [2], and in the absence of MARCO, more LPS could be expected to bind to CD14 and signal more pro-IL-1β production [24, 25]. In order to rule out the possibility that the differences in IL-1β release in the absence of MARCO was due to increased availability of LPS for signaling through CD14, ovalbumin (OVA) was used as an alternate stimulant to increase pro-IL-1β levels in AM [46]. As shown in Figure 3, IL-1β release with OVA as a stimulant was comparable to LPS. Furthermore, CA-074-Me was effective in blocking IL-1β release. Therefore, the results with OVA and the
cathepsin B inhibitor indicate that the difference in IL-1β maturation in MARCO<sup>−/−</sup> and WT, was not due to differences in availability of LPS acting on CD14 and that regardless of the source of signal 1 (LPS or OVA) that inhibiting cathepsin B blocked IL-1β maturation and release.

*Effect of silica on Caspase-1*

Measuring IL-1β release is a proxy measure of NLRP3 inflammasome activation since it requires both signal 1 (NF-κB mediated pro-IL-1β generation) and signal 2 (NLRP3 inflammasome activation). Therefore, a more direct assay for NLRP3 inflammasome activation is to measure caspase-1 activity. Primary AM from MARCO<sup>−/−</sup> and WT were stimulated with or without LPS and then treated with silica (100 µg/ml) for 4 hrs prior to caspase-1 assay. The pattern of results for Caspase-1 activity was similar to IL-1β release in that it was higher in MARCO<sup>−/−</sup> AM compared to WT AM (Figure 4). Therefore, the results suggest that the effect of MARCO on the NLRP3 inflammasome or signal 2 pathway and does not involve signal 1, consistent with the above findings using OVA as a signal 1 initiator. Furthermore, the findings are consistent with the cathepsin B results, which also suggested that the effect of MARCO on modulating the NLRP3 inflammasome pathway. Taken together, the results suggest that the target of the contribution of MARCO in regulating IL-1β release with silica may well be at lysosomal membrane permeability since in the absence of MARCO there is less silica being taken up although there is more cathepsin B release and correspondingly greater Caspase-1 activity.

*Induction of pro-IL-1β expression in AM*

Pro-IL-1β mRNA expression in MARCO<sup>−/−</sup> and WT AM was measured in order to evaluate the contribution of MARCO to signal 1 activation. Primary AM from MARCO<sup>−/−</sup> and WT mice were treated with
LPS, silica, or left untreated. Pro-IL-1β expression in AM was measured by RTPCR and the results are shown in Figure 5. Pro-IL-1β expression was higher in MARCO<sup>+/−</sup> AM compared to WT following LPS stimulation. This finding is consistent with the increased binding of LPS to CD14 in the absence of MARCO, although as the results presented in Figure 4 illustrated primary contribution of MARCO was to regulate signal 2. Silica treatment produced negligible expression of pro-IL-1β in MARCO<sup>+/−</sup> and WT AM tested. These results confirm that LPS is a stimulant for pro-IL-1β expression, while silica exposure has minimal to no effect on pro-IL-1β expression. Consequently, activation of signal 2 has little impact on signal 1.

Effect of inflammasome activation on IL-6 release

In order to evaluate whether the effect of MARCO was specific (restricted to inflammasome cytokines) or more global on macrophage cytokine production, the effect of silica on MARCO<sup>+/−</sup> and WT AM IL-6 production, which does not require NLRP3 inflammasome activation was also measured after 24 hrs (Figure 6). In contrast to the IL-1β production results, IL-6 levels in the LPS-treated group in MARCO<sup>+/−</sup> AM were significantly lower than WT AM. There was also significantly less IL-6 in the LPS- and silica-treated groups, compared to LPS stimulated group. These results are consistent with the known ability of LPS to stimulate IL-6 production and IL-6 production does not depend on inflammasome activation. However, IL-6 production is not upregulated by MARCO.

Effect of silica on acid sphingomyelinase activity

Acid sphingomyelinase is a lipid-metabolizing enzyme localized in lysosomes [36]. Acid sphingomyelinase hydrolyses membrane phospholipid sphingomyelin to ceramide [44]. In addition acid sphingomyelinase is an important regulator of the sphingolipid metabolism pathway [43].
sphingomyelinase levels were measured following silica treatment of AM because of its reported association with inflammation and acute lung injury [20]. Primary AM from MARCO−/− and WT mice were treated with or without silica (100 µg/ml) for 1 hr. After 1 hr cell lysates were prepared for sphingomyelinase activity and the results are shown in Figure 7. There was a significant increase in acid sphingomyelinase activity in MARCO−/− AM treated with silica compared to WT AM incubated with silica. These results indicate that acid sphingomyelinase activity was significantly higher in MARCO−/− AM than WT supporting the notion that silica exposure affects the sphingolipid pathway and consequently may decrease sphingomyelin and increase ceramide levels in the absence of MARCO [36].

*Effect of silica on intracellular cholesterol*

The results above demonstrated that silica exposure impacted lipid metabolism, which is associated with lysosomes. Since the critical impact of silica signaling appears to be on lysosomal membrane permeability and cholesterol is known to affect lysosomal integrity [45] the potential role of cholesterol content in AM was examined as a potential mechanistic explanation for LMP. Therefore, cholesterol levels in WT and MARCO−/− AM were assessed by loading with 1 µg/ml TopFuor cholesterol for 24 h and then treated with or without silica for 4 hrs. Figure 8 shows a significant increase in cholesterol in WT AM in response to silica exposure compared to control. However, intracellular cholesterol in MARCO−/− AM in response to silica exposure did not change significantly compared to control. This result suggested that the total intracellular cholesterol level was increased in WT AM following silica exposure in an effort to help stabilize phagolysosomes to prevent LMP following silica exposure. In contrast, AM lacking MARCO were not able to take up as much cholesterol making them more susceptible to LMP.
Effect of lysosomal cholesterol on NLRP3 inflammasome

The above results suggested that cholesterol in the lysosomal membrane can influence lysosomal membrane stabilization, inflammasome activation and downstream inflammation. In order to confirm the role of cholesterol on silica-induced LMP leasing to IL-1β release, the cholesterol trafficking modifier U18666A was used. U18666A blocks the intracellular trafficking of cholesterol and the exit of free cholesterol from the late endosomal compartment [42]. It also inhibits oxidosqualene cyclase and desmosterol reductase eliminating cholesterol biosynthesis [42]. Primary AM from WT mice were treated with U18666A for 24 hrs and then exposed to silica followed by LPS for another 24 hrs. Cell viability was also measured at 24 hrs post-particle exposure by the MTS assay. Silica caused a significant decrease in cell viability; however, there was significant increase in cell viability in silica-U18666A exposed group compared to baseline silica group demonstrating protection against cell death (Figure 9A). There was the expected significant IL-1β release following silica exposure (Figure 9B) compared to control. However, IL-1β release was significantly less in the silica-U18666A exposed group compared to silica treatment alone. Taken together, these results indicate that lysosomal cholesterol accumulation can decrease LMP, NLRP3 inflammasome activation and IL-1β release.
Figure Legends

Figure 1. *Evaluation of the effects of doses of silica and IL-1β production in AM from WT and MARCO-/mice.* (a) Dose response to silica-stimulated IL-1β release from AM co-cultured with endotoxin (LPS at 20 ng/ml) 24 hrs was measured by ELISA. Mean ± SEM IL-1β from cultured macrophages where filled circle ● indicates MARCO⁺/⁻ AM and open circle ○ indicates C57BL/6 WT AM. Double asterisk ** indicates P < 0.01 compared to WT control at the corresponding concentration, n = 4 per experimental group. (b) IL-1β release from WT AM with MARCO Ab. AM were pretreated with or without MARCO Ab (5 µg/ml) then exposed to silica (100 µg/ml) with or without LPS (20 ng/ml) 24 hrs. Mean ± SEM IL-1β release from cultured macrophages was analyzed by ELISA. Triple asterisk *** indicates P < 0.001 compared to control at the corresponding treatment, n = 4 per experimental group. Unstimulated cells were used as control.

Figure 2. *Cathepsin B activation in MARCO-/⁻ and WT AM exposed to silica.* MARCO⁻/⁻ AM and WT AM were stimulated with or without LPS (20 ng/ml) and then exposed to silica (100 µg/ml) for 2 hrs prior to cathepsin B activity assay. Mean ± SEM cathepsin B activity where white bar indicates WT AM and grey bar indicates MARCO⁻/⁻ AM. # indicates P < 0.05 compared to MARCO⁻/⁻ unstimulated (cells only) group.
Figure 3. Effect of MARCO on NLRP3 activation using IL-1β as an indicator and OVA as signal 1. IL-1β release from WT, and MARCO⁻/⁻ AM in response to silica (100 µg/ml) co-cultured with OVA (10 mg/ml) and with or without Cathepsin B inhibitor (10 µM CA-074-Me). Mean ± SEM IL-1β from cultured macrophages where white bars indicate WT AM, grey bars indicates MARCO⁻/⁻ AM. ** indicates P < 0.01 compared to WT, n = 3 per experimental group.

Figure 4. Caspase-1 activation in MARCO⁻/⁻ and WT AM exposed to silica. Caspase-1 activation in WT and MARCO⁻/⁻ AM, was measured 4 hrs after treatment with silica (100 µg/ml) with or without LPS (20 ng/ml) as described in Methods. Mean ± SEM white bars indicate WT AM and grey bars indicate MARCO⁻/⁻ AM. Double asterisk ** indicates P < 0.01 and triple asterisk *** indicates P < 0.001 compared to WT at corresponding treatment. ### indicates P < 0.001 compared to control in the same mice strain. n = 3 per experimental group.
Figure 5. *Pro-IL-1β* mRNA expression in WT and MARCO−/−AM analyzed using RT-PCR. White bars indicate WT AM, grey bars indicate MARCO−/− AM. Fold change in pro-IL-1β expression was compared to unstimulated control.

![Figure 5. Pro-IL-1β mRNA expression in WT and MARCO−/−AM analyzed using RT-PCR. White bars indicate WT AM, grey bars indicate MARCO−/− AM. Fold change in pro-IL-1β expression was compared to unstimulated control.](image)

Figure 6. *Effect of inflammasome activation on IL-6 release.* IL-6 release from WT and MARCO−/− AM in response to silica (100 µg/ml), LPS (20 ng/ml) or silica and LPS. Mean ± SEM IL-6 from cultured AM where white bars indicate WT AM, grey bars indicate MARCO−/− AM. Triple asterisk *** indicates P < 0.001 compared to WT at corresponding treatment, ### indicates P < 0.001 compared to control in the corresponding mouse strain, n = 3 per experimental group.

![Figure 6. Effect of inflammasome activation on IL-6 release. IL-6 release from WT and MARCO−/− AM in response to silica (100 µg/ml), LPS (20 ng/ml) or silica and LPS. Mean ± SEM IL-6 from cultured AM where white bars indicate WT AM, grey bars indicate MARCO−/− AM. Triple asterisk *** indicates P < 0.001 compared to WT at corresponding treatment, ### indicates P < 0.001 compared to control in the corresponding mouse strain, n = 3 per experimental group.](image)
Figure 7. *Effect of silica on acid sphingomyelinase activity.* Acid sphingomyelinase activity in WT and MARCO−/− AM, 1 hr after treatment with silica (100 µg/ml). White bars indicate WT AM and grey bars indicate MARCO−/− AM. Triple asterisk *** indicates $P < 0.001$ compared to WT at corresponding treatment. ### indicates $P < 0.001$ compared to control at the corresponding mouse strain, $n = 3$ per experimental group.

![Graph showing effect of silica on acid sphingomyelinase activity](image)

Figure 8. *Increased cholesterol uptake in WT AM in response to silica.* Isolated AM were loaded with TopFluor cholesterol (1 µg/ml) for 24 hrs prior to silica (50 µg/ml) exposure in RPMI media with 5% delipidated FBS. Silica exposure was 4 hrs in regular RPMI culture media. The wells were read in a microplate reader at 488 nm ex/525 nm em. Asterisk * indicates statistical significance at $P < 0.05$ compared to baseline WT response, $n = 7$.

![Graph showing increased cholesterol uptake in WT AM](image)
Figure 9. *Cholesterol trafficking modifier U18666A decreases WT AM response to silica (50 µg/ml).* (a) Cell viability at 24 hrs post-particle exposure by MTS assay. (b) IL-1β release 24 hrs following silica exposure co-cultured with LPS (20 ng/ml) to stimulate NF-κB activation. Asterisks *** indicate statistical significance at $P < 0.001$ compared to corresponding control condition. Hash tags ### indicate significance at $P < 0.001$ compared to baseline silica-exposed response, $n = 3$. 
**Experimental Section**

**Mice**

Breeding pairs of C57Bl/6 were originally purchased from the Jackson Laboratory (Bar Harbor, ME); while MARCO null breeders were kindly provided by Dr. Lester Kobzik (Harvard School of Public Health, Boston, MA). MARCO null mice were on C57BL/6 background. Animals were housed in micro isolators on a 12-hr light/dark cycle. The mice were maintained on an ovalbumin-free diet and given deionized water ad libitum. Age matched (6-8 week) male and female mice were used in all studies. All mice were maintained in the University of Montana specific pathogen free laboratory animal facility. The University of Montana Institutional Animal care and use committee approved all animal procedures.

**Particles**

Crystalline silica (Min-U-Sil-5, average particle size 1.5-2 μm in diameter), was obtained from Pennsylvania sand glass corporation (Pittsburgh, PA), was acid washed in 1 M HCl at 100° C for 1 hr. The silica was washed in sterile water three times and dried in an oven at 200° C to remove all water. A stock suspension of 5 mg/ml in phosphate-buffered saline (PBS) was made. Silica suspensions were sonicated for 1 min at half max power in a Masonix cup-horn sonicator (XL2020, Farmingdale, NY) attached to a ThermoForma circulating water-bath at 550 watts and 20 Hz (8000 Joules).

**Alveolar macrophage isolation and culture**

Mice were euthanized by a lethal injection of Euthasol™ (Virbac Corp, Fort Worth, TX). The lungs were removed with the heart. The lungs were lavaged five times with 1 ml of cold PBS. Pooled cells were centrifuged at 1500 x g for 5 min. The lavage fluid was aspirated and discarded. The cells were resuspended
in 1 ml of RPMI 1640 culture media supplement with 10% fetal bovine serum, 100 IU penicillin, 100 µg/ml streptomycin (Media tech, Inc., Herdon, VA). Total nucleated lavage cell fractions were counted by lysing the red blood cells with Zap-globin reagent II lytic reagent (Beckman Coulter, Fullerton, CA) and by using Z2 Coulter particle count and size analyzer (Beckman Coulter, Fullerton, CA). The concentration of cells was then adjusted to 10^6 cells/ml before doing any further steps. Later the cells were treated with or without silica or endotoxin or a combination of the two. The cells were cultured in a cell culture plate overnight at 37° C in a water-jacketed CO₂ (5%) incubator (ThermoForma, Mariette, OH). For determining specificity of MARCO in IL-1β release experiment, AM from WT mice were treated with MARCO Ab (polyclonal goat IgG, rmMARCO; aa 70-518 accession number: Q60754, Catalog number: AF2956 obtained from R & D Systems, Minneapolis, MN) 30 min prior to exposure to silica or endotoxin for 24 hr. IL-1β was measured as described below.

**Cytokine Assays**

Culture supernatants were assayed for cytokines with commercially available kits according to the manufacturer’s protocol. IL-1β measurements were determined by using Duo-set kits (BD and R&D systems). IL-6 measurements were determined using Duo-set kits (R&D systems). Colorimetric analysis was done using a Spectra Max 190 plate reader (Molecular Devices, Sunnyvale, CA) at 450 nm. Data are expressed as mean ± SEM picograms/ml of retrieved culture supernatant.

**RNA isolation**

RNA Isolation was performed using Trizol according to the manufacturer’s protocol (Trizol Reagent, Invitrogen, Carlsbad, CA). Further purification was performed using the E.Z.N.A. Total RNA Kit I according to the RNA cleanup protocol from Omega Bio-Tek, Inc. (Norcross, GA). The final elution step was
performed with 40 μl of RNase free water. RNA quantity was determined using a NanoDrop ND 1000 Spectrophotometer (NanoDrop Technologies, LLC, Wilmington, DE).

\textit{cDNA production} cDNA production was performed according to the qScript cDNA protocol from Quanta BioSciences (qScript cDNA SuperMix, Gaithersburg, MD).

\textit{Real time PCR}

Pro IL-1β message expression was measured using a Stratagene Mx3005p instrument with MxPro software (Agilent Technologies, Santa Clara, CA). Primers (Integrated DNA Technologies, Coraville, IA, USA) were as follows: β-actin forward sequence (5’-ACACTGTGCCCCATCTACGAG-3’), reverse sequence (5’-TCAACGTCACACTTCATGATG-3’), IL-1β primers were derived from IL-1β sequence (accession number: NM_008361.3), forward primer sequence: (GGTACATCAGCACCTCACAA), reverse primer sequence (TTAGAAACAGTCCAGCCCATAC). Reactions were performed using FastStart Universal SYBR Green Master (ROX) mix (Roche Diagnostics Corp., Indianapolis, IN) according to manufacturer’s protocol. Expression levels were normalized to Actb expression, and fold changes were calculated using the \(\Delta\Delta C_t\) method.

\textit{Acid sphingomyelinase assay}

Cell lysates were prepared from alveolar macrophages using a Dounce homogenizer. Cell lysates were assayed for acid sphingomyelinase with commercially available kits (K-3200 Echelon biosciences Inc., Salt Lake City, UT 84108) according to the manufacturer’s protocol. Colorimetric analysis was done using a Gemini XS plate reader (Molecular Devices, Sunnyvale CA) at 360 nm excitation and 460 nm emission. Data are expressed as pmol/hour of active acid sphingomyelinase.
**Cathepsin B activation assay**

Isolated AM were used to determine cathepsin B activation. Cathepsin B activation was determined in the AM after 2 hr of incubation with silica, silica and LPS, LPS and unstimulated cells. Cathepsin B activity was determined using Magic Red Cathepsin B Detection Kit (ImmunoChemistry Technologies, Bloomington, MN 55431). The Gemini XS plate reader was used to analyze cathepsin B activation at 590 ex and 630 em (Molecular Devices, Sunnyvale CA).

**Caspase-1 activation assay**

Caspase-1 activation was determined in isolated AM after 4 hr of incubation with silica, silica and LPS, LPS and non-treated cells. Caspase-1 activity was determined using FAM-FLICA in vitro Caspase-1 kit (ImmunoChemistry Technologies, LLC, Bloomington, MN 55431). The Gemini XS plate reader was used to analyze caspase-1 activation at 492 ex and 520 em (Molecular Devices, Sunnyvale, CA).

**TopFluor cholesterol loading experiments**

Isolated AM from C57BL/6 WT and MARCO−/− mice (process described above) were cultured at 100 x 10^3 cells per well in 96-well tissue culture plates for 24 hrs prior to silica exposure. The cells were cultured with 1 μg/ml TopFuor cholesterol (Avanti Polar Lipids, Alabaster, AL) in RPMI media with 5% dilapidated FBS. After the 24 hr loading period, the cells were switched to normal media described above and exposed to silica at 50 μg/ml for 4 hr. The plates were read in a Gemini fluorometric plate reader (Molecular Devices, Sunnyvale CA) at 488 ex and 525 em.
**U18666A cholesterol modification experiments**

Isolated AM from C57BL/6 WT mice (process described above) were cultured at 100 x 10^3 cells per well in 96-well tissue culture plates for 24 hr ± 1 μM U18666A (Sigma, St Louis, MO) prior to silica exposure. U18666A is an amphipathic steroid 3-β-[(2-(diethyl amine) ethoxy] androst-5-en-17-one. It blocks the intracellular trafficking of cholesterol and the exit of free cholesterol from the late endosomal compartment. The mechanism of action for the accumulation of cholesterol in late endosomes and lysosomes can be attributed to the amphipathic property of the compound [42]. U18666A also inhibits oxidosqualene cyclase and desmosterol reductase eliminating cholesterol biosynthesis [42]. After the 24 hr loading period, the cells were exposed to silica at 50 μg/ml, in addition to a 20 ng/ml LPS co-culture, for another 24 hr. The LPS was used to stimulate NF-κB, leading to pro-IL-1β production. The culture media was retrieved for IL-1β assay (R & D Systems, Minneapolis MN) and the cells were tested for viability using a MTS assay according to the manufacturer’s instructions (Promega, Madison, WI).

**Statistical Analysis**

Statistical analyses involved comparison of means using a one or two-way ANOVA followed by Dunnett’s test or Bonferroni’s test to compensate for increased type I error. All probabilities were two-tailed unless otherwise stated. Statistical power was greater than 0.8. Statistical significance was defined as a probability of type I error occurring at less than 5% (P< 0.05). The minimum number of experimental replications was 3 – 4 depending on the experiment. Graphics and analyses were performed on PRISM 6.0.
Conclusions

This study focused on understanding the role of MARCO in inflammation on silica exposure. In the present study we demonstrated that in the absence of MARCO receptor or when MARCO Ab was used to block MARCO receptor function, silica increased NLRP3 inflammasome activation and pro-inflammatory cytokine release in AM from MARCO\textsuperscript{-/-} mice compared to AM from WT mice. Exposure to silica caused more LMP and greater cathepsin B release in MARCO\textsuperscript{-/-} AM compared to WT AM. These results suggested that the absence of MARCO enhanced AM susceptibility to silica at the level of LMP. The mechanism of the increased LMP in the absence of MARCO was attributed to changes in cholesterol trafficking. In WT AM silica treatment resulted in increased cholesterol uptake that was reduced in the absence of MARCO. Furthermore, blocking cholesterol trafficking with U186666A greatly decreased silica-induced cytotoxicity and inflammasome activation. Taken together, these results demonstrate the important role that cholesterol plays in phagolysosomal stability and suggests a potential therapeutic site to decrease particle-induced inflammation.
References in Chapter 3


41. Hentze, H; Lin, X.Y; Choi, M.S.K; Porter, A.G; Critical role for cathepsin B in mediating caspase-1-dependent interleukin-18 maturation and caspase-1-independent necrosis triggered by the microbial toxin nigericin. Cell Death and differentiation, 2003, 10:956-968.


43. Huang, Y.L; Huang, W.P; Lee, H; Roles of sphingosine 1-phosphate on tumorigenesis. World J Biol Chem. 2011 Feb, 2:25-34.


Chapter 4 - Anti-inflammatory effect of Imipramine in silica-induced inflammation

Abstract

Background

Inhalation of crystalline silica is associated with pulmonary inflammation and silicosis. Although silicosis remains a prevalent health problem throughout the world, effective treatment choices for silicosis are limited. Imipramine is a FDA approved tricyclic antidepressant drug that has lysosomotropic characteristics and has recently emerged as a potential anti-inflammatory agent. The aim of this study was to determine the potential of imipramine to inhibit pulmonary inflammation following silica exposure.

Results

Studies used isolated alveolar macrophages from C57Bl/6 mice, and acute and chronic in vivo C57Bl/6 mouse silica exposure model. The results demonstrated that in vitro pretreatment with imipramine significantly attenuated silica-induced cytotoxicity, lysosomal release of cathepsin B and release of mature IL-1β from alveolar macrophages, which indicated inhibition of NLRP3 inflammasome activation. Pretreatment with imipramine in vivo reduced silica-induced inflammation (evident from the decrease in the chemokine KC and neutrophil infiltration) in an acute model. Furthermore, imipramine was effective in blocking chronic inflammation and fibrosis in a long-term model of silicosis.

Conclusions

Taken together, imipramine demonstrated anti-inflammatory potential against the silica exposure in vitro and in vivo. The results suggest that the mechanism of action for imipramine is most likely the prevention of phagolysosomal lysis. Thus, imipramine could be a potential protective agent for silica-induced inflammation and subsequent disease progression.
Background

Silicosis is a lung disease characterized by progressive lung inflammation and fibrosis, remaining a prevalent health problem throughout the world (Administration, 2002a, Administration, 2002b, Parks et al., 1999). The seriousness of the disease is reflected by the morbidity and disabling illnesses that continue to occur among the workers exposed to crystalline silica (Administration, 2002b, Network, 2007). Currently, treatment choices for silicosis are limited and accompanied by complex adverse outcomes (Network, 2007). Therefore, it is crucial to determine the mechanism of silica-induced inflammation and disease progression, before more targeted potential therapeutics can be developed.

When respirable particulates of 2.5 microns or less deposit in the pulmonary alveolus, they are cleared by alveolar macrophages (AM) and subsequently contained in phagosomes (Moretti and Blander, 2014). The lysosomes fuse with the phagosomes forming phagolysosomes and deliver lysosomal enzymes to degrade any phagocytosed particulates (Moretti and Blander, 2014). The lysosome lumen has an acidic pH of 4.5, which is maintained by an ATP-dependent proton pump (Hamilton et al., 2008, Guicciardi et al., 2004). The lysosomal membrane is protected from the acidic hydrolases by lysosome specific expression of membrane proteins such as LAMP-1 and LAMP-2 (Lysosome Associated membrane Proteins), which are heavily glycosylated and resist digestion by the lysosomal hydrolases (Hamilton et al., 2008, Cesen et al., 2012). However, a number of particulates including silica cannot be degraded which results in lysosomal membrane permeabilization (LMP) (Boya and Kroemer, 2008, Guicciardi et al., 2004, Cassel et al., 2008, Hornung et al., 2008). However, the exact cause of particle-induced lysosomal instability is not clearly understood.
LMP results in the release of lysosomal proteases, including cathepsin B, to the cytoplasm (Eskelinen, 2006, Johansson et al., 2010, Cesen et al., 2012). Cathepsin B in the cytosol triggers assembly of the multiprotein complex, NLRP3 (Nucleotide-binding domain, and Leucine-rich Repeat containing family, Pyrin domain containing) inflammasome (Hentze et al., 2003). NLRP3 inflammasome assembly leads to cleavage of the CARD domain of pro-Caspase-1 resulting in activation of Caspase-1 (Schroder and Tschopp, 2010). However, another signal is required to achieve cytokine release, which can be triggered by endotoxin (lipopolysaccharide or LPS), to activate the NF-κB pathway. NF-κB is responsible for formation of pro-IL-1β and pro-IL-18 (Cassel et al., 2008, Franchi et al., 2009). Active Caspase-1 cleaves pro-IL-1β and pro-IL-18 to their active and secreted forms, IL-1β and IL-18, respectively (Schroder and Tschopp, 2010). IL-1β and IL-18 have been closely associated with inflammation and development of lung fibrosis (Johansson et al., 2010, Cassel et al., 2008, Pedra et al., 2009).

Since LMP plays a critical and regulatory important role in NLRP3 inflammasome activation and subsequent inflammation, LMP could be an important target for novel therapeutics for the treatment of inflammatory diseases linked to lysosomal instability. For example, a drug or an agent that can stabilize the lysosome and prevent membrane permeabilization may have the potential to attenuate downstream inflammation. A potential anti-LMP agent is imipramine (IMP), which has lysosomotropic properties (MacIntyre and Cutler, 1988), and is a FDA approved tricyclic antidepressant drug. Pretreatment with imipramine, has been reported to attenuate the inflammatory response and improve survival in an endotoxin-induced acute lung model (Huang et al., 2011, Leventhal et al., 2001). In another study the authors reported that imipramine and surfactant synergistically suppressed acid sphingomyelinase (A-SMase) activity in lung tissue and reduced ceramide generation and improved pulmonary function in a newborn piglet lavage model (von Bismarck et al., 2008). However, imipramine has not been examined in models of particulate-induced
inflammation. Therefore, the potential of imipramine to inhibit inflammation following silica exposure was examined in this study.

We hypothesized that a lysosomotropic drug like imipramine will stabilize lysosomes, thereby decreasing NLRP3 inflammasome activation and inhibit downstream inflammation. *In vitro* studies were conducted using AM from C57Bl/6 mice to demonstrate that imipramine affected LMP and decreased NLRP3 inflammasome activation. Since silicosis develops in humans due to exposure to crystalline silica over an extended period, we used C57Bl/6 mice to simulate human exposure. Therefore, *in vivo* studies with C57Bl/6 mice were conducted to determine if imipramine could decrease silica-induced acute inflammation. Further, we determined the effect of imipramine as a protective and a therapeutic agent in a long-term crystalline silica exposure model.

**Results**

*Effect of imipramine on silica-induced NLRP3 inflammasome activation in vitro*

AM isolated from C57Bl/6 WT mice were used to evaluate the anti-inflammatory response of IMP on NLRP3 inflammasome activation in primary AM. Isolated AM were treated ± IMP for 30 min and then exposed to LPS (20 ng/ml) only or silica (100 µg/ml) plus LPS. The results in Figure 1A show the expected significant increase in IL-1β release in the silica plus LPS-exposed cells compared to the control cultures. IMP pretreatment significantly inhibited IL-1β release following silica and LPS exposure. Similar results were obtained with transformed human THP-1 cells (data not shown). These results demonstrate that IMP can significantly block silica-induced NLRP3 inflammasome activation and IL-1β release from macrophages.
In order to determine whether the imipramine-induced decrease in IL-1β release was due to any unexpected toxicity of imipramine, cytotoxicity of C57Bl/6 WT AM in the presence or absence of IMP and silica was evaluated as shown in Figure 1B. Isolated C57Bl/6 AM were pretreated ± IMP for 30 mins then exposed to silica overnight. AM exposed to silica showed the expected significant loss in cell viability (cytotoxicity) compared to baseline control. However, IMP was effective in significantly preventing silica-induced cell toxicity. Consequently, IMP blocked both the silica-induced NLRP3 inflammasome mediated IL-1β release and cytotoxicity.

A potential explanation for imipramine protection of AM cytotoxicity could be due to inhibition of silica uptake reducing the amount of silica in phagosomes and potential contribution to LMP. Therefore, the effect of IMP on silica uptake was measured by side scatter and data shown in Figure 1C. C57Bl/6 AM were pretreated with ± IMP for 30 mins and then exposed to silica for 90 mins before side scatter was measured by flow cytometry. As anticipated, AM exposed to silica showed significant particle uptake (measured as an increase in side scatter) regardless of IMP pretreatment (i.e., IMP pretreatment did not affect silica uptake). Taken together, the in vitro data suggested that IMP inhibited both silica-induced NLRP3 inflammasome activity and cell viability without affecting silica uptake mechanisms. This indicated that IMP most likely had fairly specific effects on AM phagolysosomal stability.

*Effect of imipramine on phagolysosomal stability*

Silica exposure is proposed to cause permeability of the phagolysosomal membrane, leading to the release of lysosomal enzymes (e.g., cathepsin B) to the cytoplasm. Cathepsin B is associated with NLRP3 inflammasome activation (Hentze et al., 2003). In order to evaluate whether IMP can affect phagolysosomal
stability, AM from C57BL/6 WT mice were pretreated ± IMP and exposed to silica for 2 hr. The presence of active cathepsin B was determined by adding fluorescent cathepsin B substrate to AM 45 min prior to analysis and cathepsin B activity was analyzed using confocal microscopy. In addition, lysotracker green was added concomitantly with the cathepsin B substrate to fluorescently label the intact acidic lysosomes. These results are shown in Figure 2 as confocal images at 600x magnification. With the exception of the silica-exposed cells (Figure 2B), co-localization of cathepsin B (red) and acidic lysosomes (green) is evident by speckled yellow staining within the cells’ cytoplasm (Figures 2A, 2C and 2D). In contrast, the silica-exposed cells show only green speckles (intact acidic lysosomes) within the cell cytoplasm indicating the absence of co-localized cathepsin B (red), consistent with silica-induced phagolysosomal permeability.

This can be further illustrated by showing the same culture conditions in the absence of lysotracker green stain as seen in Figure 3. Figure 3B, the silica-exposed cells, clearly shows most cells with a diffuse red stain throughout the cytoplasm. In contrast, Figures 3A, C and D show the cathepsin B (red) as contained within an organelle structure, presumably the phagolysosome. Imipramine pretreatment was effective in decreasing phagolysosomal permeability in the silica-treated cells (Figures 2D and 3D), as the cells appear more like the control conditions where the silica was absent. This result supported the hypothesis, that the mechanism of imipramine inhibition of NLRP3 inflammasome activity and cytotoxicity was stabilization of the phagolysosome resulting in sequestration of cathepsin B.

**Effect of imipramine on acid sphingomyelinase (A-Smase) activity**

Sphingomyelin is hydrolyzed to ceramide and phosphorylcholine by A-SMase at an acidic pH in the lysosomes (Leventhal et al., 2001, Huang et al., 2011). Increased ceramide production has been indicated in inflammation in response to a large variety of stressors (Leventhal et al., 2001). An increase in A-SMase activity has also been implicated in inflammation, acute lung injury and other pathological conditions.
(Canals et al., 2011, Dhami et al., 2010). Therefore, A-SMase activity was measured in C57Bl/6 AM as described in Methods. A-SMase activity in the silica-exposed AM was similar to the no imipramine control (Figure 4), suggesting that silica did not increase A-SMase activity. However, imipramine exposed control AM and imipramine pretreatment of silica-exposed AM significantly inhibited A-SMase activity. This result indicated that imipramine is effective in inhibiting A-SMase activity consistent with the literature (von Bismarck et al., 2008, Yang et al., 2010, Goggel et al., 2004, Kornhuber et al., 2010, Sakata et al., 2007). However, silica had no effect on A-SMase activity of WT AM suggesting that increased A-SMase activity might not be part of the mechanism for silica-mediated LMP.

Silica-induced inflammatory response in-vivo

Since imipramine was effective in decreasing silica-induced IL-1β release from macrophages in vitro, it suggested that imipramine could have potential to be anti-inflammatory in vivo. Therefore, C57BL/6 WT mice were given imipramine (25 mg/kg) or vehicle (100 µl) by intraperitoneal (IP) injection 30 min before silica (1 mg/kg) or PBS (25 µl) exposure. After 24 hr of exposure, whole lung lavage was performed and cells from the lavage fluid were centrifuged and isolated from the lavage fluid. Lavage fluid was analyzed for IL-1β and chemokines (keratinocyte-derived chemokine (KC) and monocyte chemoattractant protein-1 (MCP-1)) that are associated with neutrophil recruitment (De Filippo et al., 2008). The results are shown in Figure 5A. As expected, there was significant increase in IL-1β in the lavage fluid in the silica-treated group, whereas imipramine treatment decreased IL-1β. Though the imipramine treatment was not statistically significant, it followed the same pattern as observed in vitro.

Silica treatment also significantly increased the expression of KC and MCP-1 in the lavage fluid caused an opposite effect on MCP-1 expression. Imipramine significantly increased silica-induced MCP-1
expression in lung lavage fluid. The results of neutrophil infiltration after 24 hr post exposure to silica or vehicle with or without imipramine pretreatment are shown in Figure 5D. In the silica-exposed group there was a significant increase in neutrophil infiltration, as expected, and imipramine pretreatment significantly inhibited neutrophil infiltration following silica exposure. Therefore, taken together, imipramine was effective in blocking the acute (24 hr) inflammatory response due to silica instillation.

Silica-induced inflammasome activation in AM ex-vivo

In order to determine whether imipramine had blocked inflammasome activation in macrophages in vivo, AM were collected from the lavage fluid in the 24 hr experiment above. The lavaged cells were cultured ex-vivo for 24 hr ± LPS (20 ng/ml) in order to determine the bioactivation state of AM in vivo. The results of IL-1β release from the macrophages are shown in Figure 6. There was a significant increase in IL-1β release in the silica-exposed group compared to that in the controls. However, IMP pretreatment in vivo significantly decreased IL-1β release following silica in vivo and LPS ex vivo exposure. Therefore, IMP had direct effects on AM when given in vivo to block AM release of IL-1β. Taken together, the results indicate that the anti-inflammatory effect of IMP following silica exposure followed the same pattern in vitro, in vivo and ex-vivo.

Effect of imipramine on inflammation post-exposure to silica in the 24 hr study

In the studies above, IMP was given to C57Bl/6 mice prior to administration of silica. In order to determine whether IMP would also be effective if given after silica, C57BL/6 WT mice were first exposed to silica (1 mg/kg) or vehicle (25 µl) by oropharyngeal (OP) aspiration. After 30 min, mice were given IMP (25 mg/kg in PBS) or PBS alone (sham) by IP injection. After 24 hr, whole lung lavage was performed and cells from the lavage fluid were centrifuged and separated. Lavage fluid was analyzed for the inflammatory
cytokine IL-1β. Figure 7A shows that IL-1β was significantly higher in the group exposed to silica compared to the controls, however, imipramine treatment did not have a significant inhibitory effect on IL-1β release following exposure to silica. The lavaged cells were cultured ex-vivo for 24 hr in the presence and absence of LPS (20 ng/ml) in order to determine the bioactivation state of AM in vivo and whether IMP treatment affected AM. As expected, there was a significant increase in IL-1β release in the silica-exposed group compared to control (Figure 7B). Furthermore, IMP pretreatment in vivo significantly decreased IL-1β release from AM following silica exposure. This result implied that exposure to silica resulted in NLRP3 inflammasome activation in vivo and that treatment with IMP after exposure to silica was still able to attenuate NLRP3 inflammasome activity in isolated AM.

Histopathological analysis of lung tissues and lung fibrosis following long-term exposure to crystalline silica

The above studies demonstrated that IMP was effective in blocking acute inflammation caused by silica exposure. The next step was to determine whether continuous treatment with IMP could block chronic inflammation and fibrosis caused by silica exposure. Therefore, C57Bl/6 mice were given osmotic pump implants to continuously release IMP (or PBS sham) as described in Methods. Simultaneously, the mice were given 1 mg silica and three additional doses of 1 mg silica were given 7, 14 and 21 days later to simulate chronic exposure as previously described (Beamer and Holian, 2005). The exposure was continued until day-42 and the lungs were examined for chronic inflammation (pathology by histology) and collagen content. Figure 8 (A to D) shows representative sections from vehicle and sham-treated, vehicle and IMP-treated, silica and sham-treated, and silica and IMP-treated groups, respectively. Sections from vehicle and sham-treated, and vehicle and IMP-treated mice showed normal tissue architecture, while the silica and sham-exposed group indicated accumulation of inflammatory cells and thickening of the interstitium, which
was representative of a chronic inflammatory response. In comparison, silica and IMP-treated mice demonstrated reduced inflammatory responses.

Collagen content in the lung was measured by hydroxyproline following chronic exposure to silica and this was used to quantitate lung fibrosis. These results are shown in Figure 9. As expected, collagen levels were significantly higher in the silica-exposed group compared to the vehicle-exposed group. IMP treatment significantly attenuated collagen levels in the silica co-administered group. This result demonstrated that long-term exposure to silica lead to excess collagen deposition in the lung and that IMP attenuated the excess collagen deposition in the lungs following the silica exposure. These findings imply that IMP protected the lungs from silica-induced fibrosis and excess collagen deposition when concomitantly administered.

Potential therapeutic effect of imipramine following exposure to crystalline silica

The studies above demonstrated that IMP was able to decrease chronic inflammation and fibrosis when given at the same time as silica in a chronic silica exposure mouse model. However, to determine whether IMP could have the potential to act as a therapeutic, it would have to affect lung injury and fibrosis after the disease development. Therefore, IMP (or sham treatment) was given to mice for 6 weeks using osmotic pumps following the four weeks of silica administration. To determine the potential therapeutic effect of IMP on lung injury, protein levels were examined and the results shown in Figure 10A. Protein content in the lavage fluid results from protein leakage through the alveolar-capillary barrier, and in cases of lung injury, increased protein levels are found in the lavage fluid. Lung lavage protein levels were significantly increased in the silica and sham-treated group compared to the controls. In the silica and IMP-treated group, lung lavage protein levels were decreased compared to the silica and sham-treated group. This
result suggested that imipramine treatment following exposure to silica can attenuate silica-induced lung injury.

The effect on collagen expression is shown in Figure 10B. As expected, collagen content was higher in the silica and sham-treated group compared to the controls. However, collagen expression in the silica and IMP-treated group was not significantly elevated over the vehicle group and was reduced compared to the silica and sham-treated group. Taken together, the long-term in vivo results indicated that IMP treatment, after four weeks of silica exposure, did affect the development of silica-induced chronic lung pathology.

Discussion

Chronic exposure to silica leads to development of inflammation and fibrosis. The aim of this study was to determine the potential of imipramine to inhibit inflammation following crystalline silica exposure. Silica is phagocytized by AM, contained in the phagosome, which subsequently fuse with lysosomes. Within the phagolysosomes, crystalline silica leads to membrane permeabilization accompanied by cathepsin B release in the cytoplasm, which consequently leads to NLRP3 inflammasome assembly and Caspase-1 activation. All these are critical steps in silica-induced inflammation (Cassel et al., 2008). Therefore, phagolysosomal or lysosomal membrane permeabilization plays a significant role in the onset of inflammation via the inflammasome pathway (Hornung et al., 2008).

Our results demonstrated that silica exposure increased lysosomal protease cathepsin B activity, which correlated with increased NLRP3 inflammasome activity following silica exposure. Imipramine pretreatment attenuated cathepsin B activity following silica exposure, which suggested that imipramine acts at the lysosomal level. These results also demonstrated that pretreatment with imipramine significantly
attenuated IL-1β release, which indicated inhibition of NLRP3 inflammasome activation. The anti-inflammatory effect of imipramine was also evident in the downstream events of pro-inflammatory IL-1β cytokine release, as a result of exposure to silica. KC and neutrophil infiltration were reduced in the imipramine-pretreated group. In addition, imipramine pretreatment attenuated A-SMase activity following exposure to silica and this result was consistent with the literature (von Bismarck et al., 2008, Yang et al., 2010, Goggel et al., 2004, Kornhuber et al., 2010, Sakata et al., 2007). Taken together, these results indicate that imipramine inhibits inflammasome activation and subsequently the downstream inflammation.

Imipramine is a U.S Food and Drug Administration approved tricyclic antidepressant drug, is a potential lysosome-stabilizing agent and has lysosomotropic characteristics. Lysosomotropism is referred as the process by which an agent enters the lysosome and accumulates in the lysosomal lumen (MacIntyre and Cutler, 1988). They are lipophilic weak bases, which in a deprotonated form, crosses the lysosomal membrane, get protonated inside the lysosome resulting in the drug getting trapped inside the lysosome (MacIntyre and Cutler, 1988). Imipramine uptake in the lysosome is important because it is proposed that imipramine stabilizes the lysosome from the lysosomal lumen. Stabilization of the lysosome prevents triggering of the inflammasome activation and consequently downstream inflammation. Inside the lysosome, the lysosomotropic drugs may increase the lysosomal stability, thereby preventing the onset of inflammation by silica exposure (Reiners et al., 2011, Johansson et al., 2010, Cesen et al., 2012, Dhami et al., 2010). However the exact mechanism of action by which imipramine stabilizes the lysosomal membrane is not clear. Though there are some suggested hypotheses around imipramine’s possible mechanism of action.

Activation of A-SMase has been associated with the development and progression of several pathological conditions such as inflammation, lung injury, and fibrosis (Dhami et al., 2010, Kornhuber et al., 2010). It was observed in this study that imipramine pretreatment significantly inhibited A-SMase activity in the silica-exposed group. This result is consistent with the existing literature on the effect of imipramine on
A-SMase (von Bismarck et al., 2008, Yang et al., 2010). Inhibition of lysosomal A-SMase activity by imipramine can be a potential mechanism by which imipramine exerts its anti-inflammatory effect. A-SMase is an important lipid-metabolizing enzyme, which is normally localized in the lysosomes (Canals et al., 2011, Kornhuber et al., 2010). A-SMase hydrolyzes membrane lipid Sphingomyelin to ceramide and phosphorylcholine (Spiegel and Milstien, 2003). It has been proposed that A-SMase is bound to the intra-lysosomal membrane, thereby protecting itself against proteolytic inactivation (Yang et al., 2010, Kolter, 2011). Presence of weak bases like imipramine or any related drug in the lysosome results in the detachment of the A-SMase from the inner membrane of the lysosome and inactivation of the A-SMase by proteolytic degradation, preventing A-SMase-induced pathological conditions. However, it must be noted that heritable deficiency of A-SMase in humans results in Niemann-Pick A and B, a lysosomal storage disorder characterized by pathologic accumulation of Sphingomyelin in the cells and tissues (Maxfield and Tabas, 2005, Mukherjee and Maxfield, 2004, Ikonen and Holtta-Vuori, 2004). Drug induced inhibition of A-SMase below the basal level may result in similar pathological conditions (Lozano et al., 2001).

Another theory for anti-inflammatory effect of imipramine is its effect on the other members of the sphingolipid metabolism pathway and metabolites. Sphingolipids are bioactive lipid molecules ubiquitously present in the eukaryotic cells that provide structural integrity to the cell membranes and are important for vital cellular functions (Huang et al., 2011). Sphingolipid metabolism is associated with many critical physiological outcomes including cellular integrity, and hence, is an area of interest to understand membrane integrity and permeabilization (Canals et al., 2011). Sphingomyelin and ceramides are possible imipramine targets. Sphingomyelin is usually attached to nonesterified cholesterol before Sphingomyelin is hydrolyzed to ceramide by A-SMase and free cholesterol is released (Leventhal et al., 2001). Inhibition of A-SMase by imipramine can subsequently lead to increase in Sphingomyelin and decrease in ceramide. This may
consequently lead to an increase in nonesterified cholesterol bound Sphingomyelin in the membrane. Increasing cholesterol in the membrane can increase membrane stability and prevent lysosomal membrane permeabilization (Reiners et al., 2011). It has shown that non-esterified cholesterol content in the lysosomes regulates susceptibility to ROS-induced lysosomal membrane permeabilization (Reiners et al., 2011).

Another important target of the sphingolipid pathway is ceramide. Ceramide has been linked to apoptosis and inflammation (Huang et al., 2011, Spiegel and Milstien, 2003). However, the mechanism by which ceramide affects apoptosis and inflammation is not completely understood. It has showed that the A-SMase- dependent production of ceramide plays an essential role in the formation of lung edema and that inhibition of A-SMase dependent production of ceramide by imipramine attenuated pulmonary edema (Goggel et al., 2004). However, it is not clear whether imipramine has any direct effect on ceramide, which could account for the anti-inflammatory effects of imipramine.

It is evident that A-SMase is a key regulator of the sphingolipid metabolism pathway and that imipramine acts on the A-SMase (Kornhuber et al., 2010, Dhami et al., 2010). Furthermore, inhibiting A-SMase can improve certain pathological conditions (Yang et al., 2010, Goggel et al., 2004, Kornhuber et al., 2010). However, it is not clear whether imipramine acts exclusively by inhibiting A-SMase or whether other mechanisms are involved in the anti-inflammatory effect of imipramine. Further research needs to be done for complete understanding of the anti-inflammatory mechanism of imipramine. It will also be important to study the long-term effect of imipramine treatment in silica-induced inflammation.

The hypothesis of this study was that a lysosomotropic drug like imipramine will stabilize lysosomes, thereby decreasing NLRP3 inflammasome activation and inhibits downstream inflammation. These results
illustrated that pretreatment with imipramine significantly attenuated cathepsin B, a lysosomal protease, activity and IL-1β release, resulting from increased phagolysosomal stability and causing decreased NLRP3 inflammasome activation. Imipramine pretreatment also reduced downstream inflammation in vivo, which was evident from the decrease in the chemokine KC and neutrophil infiltration. In conclusion, an anti-inflammatory effect of imipramine was observed in vitro, ex vivo and also evident in short-term in vivo study following exposure to silica. The protective effect of imipramine on silica-induced inflammation is probably exerted through its effect on increasing phagolysosome stability.

In the present study we also determined whether imipramine has the potential to be both a protective agent and a potential therapeutic agent following chronic exposure to silica. Environmental or occupational exposure to crystalline silica leads to the development of silicosis in humans and there is a lag period between exposure and disease development; this lag period can be in between 5 to 20 years or more. Treatment for silicosis should aim at preventing the progression of the disease, reducing inflammation, decreasing fibrosis and improving quality of life. Imipramine co-administration with exposure to silica attenuated inflammation and excess collagen deposition. Imipramine may also emerge as a potential protective agent. Treatment with imipramine in our chronic silica exposure model decreased lung injury and excess collagen deposition. These results suggest that imipramine can be a potential agent in pulmonary inflammation treatment.

Conclusions

In conclusion, imipramine demonstrated anti-inflammatory effects against the inflammation caused by exposure to silica in the lungs. Anti-inflammatory effect of imipramine was evident in in vitro and in
vivo in an acute and chronic C57Bl/6 mouse exposure model. The mechanism of action for imipramine is most likely the prevention of phagolysosomal lysis. Thus, imipramine could be a potential protective agent for silica-induced inflammation and subsequent disease progression.

**Methods**

*Mice*

C57Bl/6 wild type (WT) mice were housed in controlled environmental conditions on a 12 hr light/dark cycle. The mice were maintained on an ovalbumin-free diet and given deionized water. Age matched (6-8 week) male and female mice were used in all studies. All mice were maintained in the pathogen free laboratory animal facility and all animal procedures were approved by Institutional Animal Care at the University of Montana.

*Particles*

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 1M HCl at 100°C for 1hr. The silica was then washed in sterile water three times and dried in an oven at 200°C to remove all water (several hrs). A stock suspension of 5 mg/ml in phosphate-buffered saline (PBS) was made. The resulting stock suspension was sonicated for 1 min in a Masonix cup-horn sonicator (XL2020, Farmingdale, NY) attached to a Forma circulating water-bath at 550 watts and 20 Hz (8000 Joules), to achieve dispersion of the silica in PBS.
Silica Instillations

Mice were exposed to crystalline silica or vehicle (sterile PBS, 25 µl) by oropharyngeal (OP) aspiration. Briefly, the mice were anesthetized using inhalation isoflurane and a volume of 25 µl of silica suspension (1 mg/Kg or 25 µg/25 g mouse) was delivered into the back of the throat. By holding the tongue to the side, the solution was aspirated into the lungs.

Alveolar macrophage (AM) isolation and cell culture

Mice were euthanized by a lethal injection of sodium pentobarbital (Euthasol, Virbac, Fort Worth, TX). The lungs were removed along with the heart. The lungs were lavaged five times with 1 ml of cold PBS. Pooled cells were centrifuged at 400 x g for 5 min. The lavage fluid was aspirated and discarded. The cells were resuspended in 1 ml of RPMI 1640 culture media supplemented with 10% fetal bovine serum, 1% antimycotic/antibiotic cocktail (100x solution, Mediatech, Manassas, VA). A sample of isolated cells (40 µl) was counted using a Z2 Coulter particle counter and size analyzer (Beckman Coulter, Miami, FL). Red blood cell contamination was eliminated with Zap-o-globin reagent (Beckman Coulter) prior to the counting.

AM were treated ± imipramine (final concentration of 25 µM) (Sigma, St Louis, MO). Cell treatment included no silica, endotoxin (LPS final concentration 20 ng/ml, Sigma), silica (final concentration of 100 or 50 µg/ml) and silica ± LPS. A 100 µl sample (100,000 cells) of cells were added to each well in 96-well plates for 24 hr in a 37° C water-jacketed CO₂ incubator (ThermoForma, Houston, TX).

In vivo mouse exposure

Imipramine was suspended in sterile PBS and sonicated for 1 min prior to intra-peritoneal (IP) injection. Imipramine (25 mg/kg or 625 µg/25 g mouse) in PBS at a volume of 100 µl or sham (sterile PBS
alone, 100 µl) was administered to the mice through IP injection prior to particle exposure. Silica was suspended in sterile PBS and sonicated for 1 min prior to instillation. Mice were exposed to silica or PBS vehicle (25 µl) by OP aspiration, as described above. After 1 day, the lungs were removed from a subset of exposed mice and lavaged with cold PBS as described above.

**Drug**

Imipramine was obtained from Sigma, was suspended in sterile PBS and sonicated for 1 min prior to administration in the mice. Imipramine (25 mg/kg or 625 µg/25g mouse) in a volume of 100 µl suspension in sterile PBS was prepared and administered to the mice through intraperitoneal injection in the mice (Yang et al., 2010).

**Subcutaneous osmotic pump implantation**

Osmotic pumps were obtained from Alzet Osmotic Pumps (Alzet, Cupertino, CA). Osmotic pumps were used to deliver imipramine continuously over an extended period of time (42 days). Animals were anesthetized using injectable anesthesia IP (Ketacine (80mg/kg), Putney Inc., Portland, ME, and Xylazine (12mg/kg) cocktail; total volume - 100µl). Skin over the implantation site was washed and shaved then betadine was applied. An incision was made adjacent to the mid-scapular region on the back of the animal. Hemostats were then inserted into the incision and a pocket formed by spreading the hemostats open and closed to spread the subcutaneous tissue. The pocket was large enough to allow some free movement of the pump. A sterile filled pump was inserted into the pocket with the delivery portal inserted first. The incision was then closed using vet bond (3M Animal Care Products, St Paul MN). Mice were given a dose of buprenorphine (0.05-.1 mg/kg, subcutaneous). Mice were monitored until they had recovered from the anesthesia.
**Cytokine Assays**

Culture supernatants were assayed for cytokines with commercially available ELISA kits according to the manufacturer’s protocol. Mouse IL-1β, and IL-6 measurements were determined using Duo-set kits from R&D Systems (Minneapolis, MN). Colorimetric analysis was done using a Spectra Max 190 plate reader (Molecular Devices, Sunnyvale, CA) at 450 nm. Data are expressed as pg/ml (note: pg/10^6 AM for *ex vivo* experiments) of retrieved culture supernatant.

**Toxicity Assay**

Cell viability was determined by MTS assay using the CellTiter^96^ assay (Promega, Madison, WI) according to the manufacturer’s protocol with one modification described below. This assay used a colorimetric dye read by a colorimetric plate reader (Molecular Devices). In order to avoid artifacts in the optical density values, steps were taken to remove the MTS reaction (transferring it into another plate) from the cell/particle mixture adhered to the plate bottom. The formation of bubbles was avoided and the plate was read at 490 nm. Data was transformed to a percent relative to the no particle/no imipramine control cells.

**Particle uptake**

Silica particle internalization was determined by flow cytometry using a side-scatter technique previously described (Hamilton et al., 2006). Isolated AM were cultured as described above ± IMP and ± silica for 90 min in suspension cultures using 1.5 mL microfuge tubes and end over end tumbling (Lab Quaker Shaker, Thermo Forma). The cells were centrifuged and washed once in PBS. The resulting cell pellet was resuspended in PBS and transferred to filter-top flow cytometry tubes (BD Biosciences, San Jose, CA) for analysis. Data was expressed as mean side scatter for 10^4 cells.
Cathepsin B activity with and without lysotracker green

C57Bl/6 WT AM were pretreated for 30 min ± imipramine (25 µM) and exposed to silica (100 µg/ml) for 2 hr. The presence of active cathepsin B was determined using cell-permeable, red fluorescent (555nm ex/584nm em), cathepsin B substrate (EMD Millipore, Billerica, MA). Acidic lysosomes were stained with lysotracker green (50 nM, Life Technologies, Grand Island, NY) with green fluorescence (488nm ex/520 em). Both fluors were added to the AM 45 min prior to imaging. The blue fluorescent (405nm ex/ 435nm em) HCS nuclear mask (Life Technologies, Grand Island, NY) was added 15 min before confocal microscopy analysis and imaging (Olympus FV1000, Olympus, Tokyo, Japan) at 600x magnifications.

Acid sphingomyelinase assay

Cell lysates were prepared from the alveolar macrophages using Dounce homogenizer (tight pestle). Cell lysates were assayed for acid sphingomyelinase with commercially available kits according to the manufacturer’s protocol (K-3200 Echelon Biosciences Inc., Salt Lake City, UT). Fluorescence analysis was done using a Gemini XS plate reader (Molecular Devices) at 360 nm ex and 460 nm em. Data are expressed as pmol/hour of active acid sphingomyelinase.

Histology

The lungs from each mouse were inflation-fixed through the trachea with 4% paraformaldehyde-PBS (Electron Microscopy Sciences, Hatfield, PA) and submerged in the same fixative overnight at 4°C. The lungs were washed with cold PBS, dehydrated with ethanol, and embedded in paraffin. Tissue sections (7 µm) were stained with hematoxylin and eosin (RAS Harris Hematoxylin and Shandon Alcohol Eosin) for histological analysis using a ThermoShandon automated stainer (ThermoFisher). Representative images were
captured with a Nikon E 800 microscope and Nikon DXM 1200 digital color camera (Nikon Inc., Melville, NY) using 40x and 60x objectives (400x and 600x magnifications respectively).

Hydroxyproline assay

Measuring hydroxyproline, an amino acid unique to collagen, quantitatively assessed collagen content. Briefly, the lung lobes were excised, weighed, and immediately frozen. The lung tissues were homogenized using a tissue homogenizer (Tissue Tearor model 985370, Biospec Products, Bartlesville, OK), in 1 ml of sterile PBS. An aliquot of lung homogenate was hydrolyzed in 12N HCl at 110°C for 24 hrs. The mixture was reacted with chloramine T and Ehrlich’s reagent to produce a hydroxyproline-chromophore that was quantified by spectrophotometry at 550 nm. Hydroxyproline content was determined by triplicate analysis of the samples to provide an average value.

Study design to determine the effect of imipramine in the 42 days study

Mice were randomly divided into four groups: vehicle plus sham (PBS), vehicle plus imipramine, silica plus sham (PBS), and silica plus imipramine. Mice were exposed to silica or vehicle (25 µl) by OP aspiration once a week for 4 weeks. Osmotic pumps were subcutaneously implanted in the mice that delivered imipramine or sham (PBS) to the mice throughout the entire length of the study (42 days). Silica-induced fibrosis in the lungs was assessed by hydroxyproline and chronic inflammatory response in the lung was evaluated by hematoxylin and eosin staining.
Study design to determine the effect of imipramine after generation of lung injury

For the first 4 weeks mice were exposed to silica or vehicle weekly in the absence of imipramine or sham pumps. Exposure to silica was discontinued and the mice were treated with imipramine or sham pumps for an additional 6 weeks in a similar manner as described above. After 10 weeks, the mice were sacrificed. Lung injury was assessed using measurements of total lavage protein levels and silica-induced fibrosis in the lung was assessed by hydroxyproline quantification as described above.
Statistical Analysis

Statistical analyses involved comparison of means using a one or two-way ANOVA followed by Tukey’s test to compensate for increased type I error. All probabilities were two-tailed unless otherwise stated. Statistical power was greater than 0.8. Statistical significance was defined as a probability of type I error occurring at less than 5% ($P < 0.05$). The minimum number of experimental replications was 3 – 4 depending on the experiment. Graphics and analyses were performed on PRISM 6.0.
Figures Legends

Figure 1. **In vitro assessment of NLRP3 inflammasome activation, toxicity and particle uptake of silica ± imipramine in the C57BL/6 AM** (A) Silica-induced NLRP3 inflammasome activation in vitro C57BL/6 WT AM. To evaluate the anti-inflammatory response of IMP on NLRP3 inflammasome activation in primary cells, AM isolated from C57Bl/6 WT mice were used. Isolated AM were treated with ± IMP (25 µM) for 30 min in the water-jacketed CO$_2$ incubator at 37°C and then exposed to LPS (20 ng/ml) and ± silica (100 µg/ml) overnight. Data were expressed as mean ± SEM IL-1β release. (B) Cell viability in isolated AM at 24 hrs following IMP and silica. C57BL/6 AM cells were pretreated with ± IMP (25 µM) for 30 min in the water-jacketed CO$_2$ incubator at 37°C and then exposed to ± silica (50 µg/ml) overnight. Data were expressed as mean ± SEM percent viability relative to control. Asterisks *** indicate $P < 0.001$, or ** $P < 0.01$ compared to baseline control. Hashtags ### indicate $P < 0.001$ compared to silica exposed group. Daggers ††† indicate $P < 0.001$ compared to the IMP treated control group. (C) Silica particle uptake in isolated C57BL/6 AM. C57BL/6 AM cells were pretreated with ± IMP (25 µM) for 30 min in the water-jacketed CO$_2$ incubator at 37°C and then exposed to ± silica (50 µg/ml) for 90 min. Data were expressed as mean ± SEM side-scatter (arbitrary units). Asterisks ** indicate $P < 0.01$ compared to baseline controls. For all experiments the white bars indicate IMP treatment (25 µM) and the grey bars indicate no IMP treatment. $n = 3$ per experimental group.
Figure 2. Effect of imipramine on silica-induced lysosomal leakage of cathepsin B. Cathepsin B is a lysosomal enzyme associated with NLRP3 inflammasome activation. C57Bl/6 WT AM were pretreated for 30 min with or without IMP (25 µM) & exposed to silica (100 µg/ml) for 2 hr. Presence of active cathepsin B was determined using fluorescent cathepsin B substrate (red fluorescence), in addition to lysotracker green (green fluorescence) staining for acidic lysosomes, both added to the AM 45 min prior to analysis and then analyzed by confocal microscopy. (A) Control AM (B) Silica-exposed AM (C) IMP-exposed AM (D) IMP and silica-exposed AM. Blue fluorescence is the cell nucleus stained with HCS nuclear mask.
Figure 3. Effect of imipramine on silica-induced lysosomal leakage of cathepsin B in the absence of lysotracker green. C57Bl/6 WT AM were pretreated for 30 min with or without IMP (25 µM) & exposed to silica (100 µg/ml) for 2 hr. Presence of active cathepsin B was determined using fluorescent cathepsin B substrate (red fluorescence), added to the AM 45 min prior to analysis and then analyzed by confocal microscopy. (A) Control AM (B) Silica-exposed AM (C) IMP-exposed AM (D) IMP and silica-exposed AM. Blue fluorescence is the cell nucleus stained with HCS nuclear mask.
**Figure 4. Effect of imipramine on sphingolipids.** Isolated AM from C57BL/6 WT were treated with IMP (25 µM) or remained untreated for 30 min in the water-jacketed CO₂ incubator at 37°C and then exposed to LPS (20ng/ml) only, or silica (100 µg/ml) and LPS for 2 h. Cell lysates were assayed for acid sphingomyelinase. Mean ± SEM IL-1β measured by ELISA where white bars indicate IMP treatment and grey bars indicate no IMP treatment.

**Figure 5. Silica-induced inflammatory lung response in-vivo.** The effect of 24 hr *in vivo* IMP treatment on the inflammatory response in mice was evaluated in C57Bl/6 WT mice. C57BL/6 WT mice were administered IMP (25 mg/kg) or sham (100 µl) by IP injection 30 min before silica (1mg/kg) or vehicle (25 µl) exposure by OP route. After the 24 hr exposure, whole lung lavage was performed and cells from the lavage fluid were centrifuged and separated. (A) The isolated lavage fluid was analyzed for IL-1β. Data expressed as mean ± SEM IL-1β. Asterisks ** indicate *P < 0.01 compared to vehicle-sham and vehicle-IMP groups. (B) The isolated lavaged cells were stained to differentiate AM and neutrophil cells. Data was expressed as mean ± SEM percent neutrophils. Asterisks *** indicate *P < 0.001 compared to vehicle-sham and vehicle-IMP control groups and hashtag # indicates *P < 0.05 compared to silica plus sham group. (C) Isolated lavage fluid was analyzed for the chemokine KC. Data were expressed as mean ± SEM KC. Asterisks *** indicate *P < 0.001 compared to vehicle-sham and vehicle-IMP groups and hashtags ## indicate *P < 0.01 compared to silica-sham group. (D) Isolated lavage fluid was analyzed for the chemokine MCP-1. Data were expressed as mean ± SEM MCP-1. Hashtags ## indicate *P < 0.01 compared to silica plus sham group. For all experiments, the white bars indicate IMP treatment and the grey bars indicate sham treatment. *n = 3* per experimental group.
A

![Graph representing IL-1β levels in PBS and Silica groups.](image)

**IL-1β (pg/ml)**

- **PBS**
- **Silica**

B

![Graph representing Number of PMN (x10⁵) in Sham and IMP groups.](image)

***Number of PMN (x10⁵)**

- **Sham**
- **IMP**

C

![Graph representing KC (pg/ml) in Sham and IMP groups.](image)

***KC (pg/ml)**

- **Sham**
- **IMP**

D
Figure 6. Silica-induced inflammasome activation isolated AM. C57Bl/6 WT mice pretreated with IMP (25 mg/kg) or sham (100 μl, IP) and then exposed to silica (1mg/kg) or Vehicle (25 μl) [O.P] 24 hr. Isolated AM were exposed to LPS (20 ng/ml) \textit{ex vivo} for 24 hr. Data were expressed as mean ± SEM IL-1β. The white bars indicate IMP and the grey bars indicate sham treatment. Asterisks *** indicate $P < 0.001$ compared to vehicle-sham, and vehicle-IMP control groups. Hashtag # indicate $P < 0.05$ compared to silica-sham group. $n = 4$ per experimental group.

Figure 7. Effect of imipramine on NLRP3 inflammasome post-exposure to silica. The effect of short-term \textit{in vivo} imipramine treatment on the inflammatory response in mice was evaluated in C57Bl/6 WT mice. C57BL/6 WT mice were exposed to silica (1mg/kg) or vehicle (25 μl). After 30 min of exposure the mice were given IMP (25 mg/kg) or sham (100 μl). After 24 hr whole lung lavage was performed and cells from the lavage fluid were centrifuged and separated. (A) Isolated lavage fluid was analyzed for the inflammatory cytokine IL-1β. Data were expressed as mean ± SEM IL-1β. Asterisks *** indicate $P < 0.001$ compared to vehicle-sham and vehicle-IMP groups. Double asterisks ** indicate $P < 0.01$ compared to vehicle-IMP group. (B) Isolated AM were exposed to LPS (20 ng/ml) \textit{ex vivo} 24 hr. data were expressed as mean ± SEM IL-1β. Asterisks *** indicates $P < 0.001$ compared to vehicle-sham, vehicle-IMP control groups and hashtags # indicate $P < 0.05$ compared to silica-sham group. For all experiments, the white bars indicate IMP treatment and the grey bars indicate sham treatment. $n = 4$ per experimental group.
Figure 8. Effect of imipramine on inflammatory responses and collagen levels following long-term exposure to silica in the 42 day (co-administration) study. Silica and IMP were concomitantly administered for the first 4 weeks. The chronic inflammatory response in the lung was evaluated by hematoxylin and eosin staining. Representative sections from (A) vehicle and sham, (B) vehicle and IMP, (C) silica and sham, and (D) silica and IMP groups has been presented.
Figure 9. Protective effect of imipramine on silica-induced fibrosis in the lungs following long-term exposure to silica in the 42 day (co-administration) study. Silica-induced fibrosis in the lungs was assessed by hydroxyproline quantification. Data were expressed as mean ± SEM hydroxyproline. Asterisks ** indicate $P < 0.01$ compared to vehicle-sham group and hashtags ## indicate $P < 0.01$ compared to the silica-sham group. The white bars indicate IMP treatment and the grey bars indicate sham treatment. $n = 4$ per experimental group.
Figure 10. Effect of imipramine on lung injury and collagen levels following long-term exposure to silica in the (post-administration) study. C57Bl/6 WT mice were exposed to silica or vehicle (25 µl) weekly for the first 4 weeks. Exposure to silica was discontinued and the mice were treated with IMP or sham for an additional 6 weeks. (A) Isolated lung lavage fluids were assessed for changes in total protein levels. The results were reported as mean ± SEM total protein. Asterisks *** indicate $P < 0.001$ and hashtag # indicates $P < 0.05$. (B) The effect of IMP on silica-induced fibrosis in the lungs was assessed by hydroxyproline quantification. Data were expressed as mean ± SEM hydroxyproline. Asterisks ** indicate $P < 0.01$ compared to vehicle-sham group. For both experiments, the white bars indicate IMP treatment and the grey bars indicate sham treatment. $n = 4$ per experimental group.
References in Chapter 4

1. ADMINISTRATION, O. S. A. H. 2002A. Crystalline silica exposure health hazard information. u.s. department of labor.


Chapter 5- Summary

Lysosomes are ubiquitous acidic single-membrane intracellular organelle present in all eukaryotic cells. Lysosomes were first described by de Duve and colleagues (Appelqvist, et al, 2013). The primary function of lysosomes is degradation of macromolecules, turnover of phospholipids and for this purpose, lysosomes are contain more than 50 acid hydrolases, including phosphatases, nucleases, glycosidases, proteases, peptidases, sulphatases, and lipases (De Duve, 1983 and Kazmi, et al 2013). Of the lysosomal hydrolases, the cathepsin family of proteases is the best characterized. The cathepsins are subdivided, according to their active site amino acids, into cysteine (cathepsin B, C, F, H, K, L, O, S, V, W, and X), serine (cathepsins A and G), and aspartic cathepsins (cathepsins D and E) (Turk and Stoka, 2007).

Recent advances in understanding the lysosomes have indicated the importance of the lysosomes in the cellular processes and they are now considered crucial regulators of cell homeostasis (Appelqvist, et al, 2013). Lysosomes are involved in intra and extra-cellular materials, plasma membrane repair, cholesterol homeostasis and apoptosis (Johansson, et al 2010 and Appelqvist, et al, 2013). Since many cellular functions involves the lysosomes, therefore, lysosomal dysfunction causes and are involved in many diseases (Appelqvist, et al, 2013). Lysosomal dysfunction contributes in the pathogenesis of diseases such as lysosomal storage disorders, cancer, Parkinson disease, atherosclerosis, gouty arthritis, rheumatoid arthritis (Vila, et al 2011). Lysosomal membrane permeabilization (LMP), i.e., release of cathepsins from lysosomes to the cytosol, is an important step in onset of inflammatory response. Exposure to silica leads to cathepsin B release to the cytoplasm, which plays a critical role in triggering NLRP3 inflammasome activation and consequently release of pro-inflammatory cytokine such as IL-1β (Hentze et al 2003 and Hornung et al
IL-1β is an important mediator of the inflammatory response and has been associated with inflammation and fibrosis development.

Exposure to crystalline silica for an extended period of time can lead to chronic inflammation in the lungs, followed by progressive fibrosis, escalating to silicosis development (GOHNET 2007). Silicosis cannot be cured and often lead to disability and fatality in humans exposed to crystalline silica (GOHNET 2007). Since silica is the basic component of soil, sand and most types of rocks and silica is used in various products in our daily life, exposure to silica remains prevalent and silicosis remains a prevalent health problem throughout the world predominantly in the developing countries (Rosenman et al., 2003 and GOHNET 2007)). Therefore, the focus of this study is to determine the role of lysosomes in silica-induced inflammasome activation and inflammation in absence of MARCO and to stabilize the lysosome and prevent LMP by a potential anti-LMP and lysosomotropic agent imipramine.

Previous studies have reported that Inhaled silica particles are encountered by alveolar macrophages (AM) in the lungs. The AM are the primary innate immune phagocytic cells at the air tissue interface responsible for clearance of particles through the mucociliary escalator and/or lymphatic systems (Hamilton et al., 2006). Previous studies have demonstrated that AM recognize and bind silica particles through class A scavenger receptors (SR) expressed on their surface(Sankala et al., 2002; Arredouani et al., 2006; Jozefowski et al., 2005). The class A SR family includes SR-A, and MARCO, which function primarily as phagocytic receptors binding to a variety of microbial components and can also modulate inflammatory signaling by Toll-like receptors (Takeuchi and Akira 2010; Areschoug and Gordon 2009). SR-AI, SR-AII and MARCO are associated with silica binding, and MARCO is the predominant receptor for
binding and uptake of unopsonized particles such as silica (Hamilton et al., 2006; Palecanda et al., 1999; Palecanda et al., 2001).

Previous in vivo results showed an increased inflammatory response in MARCO<sup>−/−</sup> mice compared with WT mice following 24 hrs of silica exposure (Thakur et al., 2009). There was an increase in total protein levels, total number of lavage cells and a significant increase in infiltration of immune cells such as AM, DC and neutrophils in MARCO<sup>−/−</sup> mice compared with WT mice, all indicating an increase in inflammation in MARCO<sup>−/−</sup> mice (Thakur et al., 2009). However, the mechanism to explain the increased inflammatory response in the absence of MARCO was not clear.

This study focused on understanding the role of MARCO in inflammation on silica exposure. MARCO (Macrophage Receptor with Collagenous Structure) is the predominant receptor for recognition and binding of silica particles by alveolar macrophages (AM). The present study demonstrated that Silica increased NLRP3 inflammasome activation and release of the pro-inflammatory cytokine, IL-1β, to a greater extent in MARCO<sup>−/−</sup> AM compared to wild type (WT) AM. A similar increase in NLRP3 inflammasome activation by silica was observed using MARCO Ab to block MARCO receptors in WT AM. Exposure to silica caused more LMP and greater cathepsin B release in MARCO<sup>−/−</sup> AM compared to WT AM and cathepsin B inhibitor (CA-074-Me) significantly alleviated pro-inflammatory cytokine, IL-1β release. These results suggested the critical role played by LMP in triggering silica-induced inflammation. The mechanism of the increased LMP in the absence of MARCO was attributed to changes in cholesterol trafficking. In WT AM silica exposure resulted in increased cholesterol uptake that was less in the absence of MARCO. Furthermore, blocking cholesterol trafficking with U186666A greatly decreased silica-induced cytotoxicity and inflammasome activation. The difference in sensitivity to LMP appears to be in cholesterol recycling.
since increasing cholesterol in AM by treatment with U18666A decreased silica-induced NLRP3 inflammasome activation, and cells lacking MARCO sequestered less cholesterol following silica treatment.

It is anticipated that the above mentioned study will have a significant impact on understanding of diseases linked to inflammasome activation and lysosomal instability. The inflammasome and lysosomal instability has been implicated in other chronic inflammatory diseases such as gout, rheumatoid arthritis and pulmonary fibrosis. In chapter 2 role of the inflammasome inflammasome-derived pro-inflammatory cytokine in pulmonary fibrosis was reviewed. Recent progress in inflammasome research has greatly contributed to our understanding of its role in inflammation and fibrosis development. Inflammasome assembly consequently leads to pro-inflammatory cytokines, such as IL-1β and IL-18 release, which, have been associated with development of pulmonary fibrosis. In addition, components of the inflammasome complex itself, such as the adaptor protein ASC have been associated with pulmonary fibrosis development. Evidence suggests that the fibrotic process can be reversed via blockade of pathways associated with inflammasome activity may provide hope for future drug strategies.

Since LMP plays a critical role in NLRP3 inflammasome activation and subsequent inflammation, LMP could be an important target for novel therapeutics for the treatment of inflammatory diseases linked to lysosomal instability. A drug or an agent that can stabilize the lysosome and prevent membrane permeabilization may potentially attenuate downstream inflammation. A potential anti-LMP agent is imipramine (IMP), which has lysosomotropic properties (MacIntyre and Cutler, 1988; Funk and Krise, 2012; Kaufmann and krise 2007), and is a FDA approved tricyclic antidepressant drug. Pretreatment with imipramine, has been reported to attenuate the inflammatory response and improve survival in an endotoxin-induced acute lung model (Huang et al., 2011, Leventhal et al., 2001). In another study the authors reported
that imipramine and surfactant synergistically suppressed acid sphingomyelinase (A-SMase) activity in lung tissue and reduced ceramide generation and improved pulmonary function in newborn piglet lavage model (von Bismarck et al., 2008). However, imipramine has not been examined in models of particulate-induced inflammation. Therefore, the potential of imipramine to inhibit pulmonary inflammation following silica exposure was examined in this study.

In vitro studies were conducted using isolated AM from C57Bl/6 mice to establish that imipramine affected LMP and decreased NLRP3 inflammasome activation. The results demonstrated that in vitro pretreatment with imipramine significantly attenuated cathepsin B, a lysosomal protease, activity and release of mature IL-1β from alveolar macrophages, which indicated inhibition of NLRP3 inflammasome activation. In vivo studies with C57Bl/6 mice were conducted to determine if imipramine could decrease silica-induced acute inflammation. In an in vivo acute C57Bl/6 mouse exposure model imipramine pretreatment reduced inflammation, as demonstrated by the decrease in the chemokine KC and neutrophil infiltration.

Since silicosis develops in humans due to exposure to crystalline silica over an extended period, we used C57Bl/6 mice to simulate human exposure. Further, we determined the effect of imipramine as a protective and a therapeutic agent in a long-term crystalline silica exposure model. The 6 weeks or 42 days co-administration study was conducted to evaluate the protective effect of imipramine treatment concomitant with 4 weeks of silica exposure with additional 2 weeks of imipramine treatment. Protective effect of imipramine was evident in the histopathological analysis of lung tissues and the protective effect of imipramine on silica-induced lung fibrosis assessed by hydroxyproline quantification. Furthermore, the therapeutic potential of imipramine in silica-induced chronic inflammation and fibrosis was evaluated. In the 10 weeks post-exposure study where 6 weeks of imipramine treatment followed 4 weeks of exposure to
Therapeutic potential of imipramine was observed in attenuating lung injury following chronic exposure to silica. However, this effect was not significant in silica-induced fibrosis in the lungs as assessed by hydroxyproline quantification. These results establish that the pretreatment or concomitant treatment of imipramine was effective in alleviating silica-induced lung pathology, signifying that the early and acute phase of silica-induced inflammation as the critical phase where potential therapeutics interventions could be effective.

Overall Conclusions

AM isolated from C57BL/6 wild-type (WT) and MARCO null mice were used to determine the role of MARCO in silica-induced inflammasome activation and inflammation. This project identified the role of MARCO in inflammation on silica exposure. This study demonstrated that in the absence of MARCO receptor or when MARCO Ab was used to block MARCO receptor function, silica increased NLRP3 inflammasome activation and pro-inflammatory cytokine release in AM from MARCO-/- mice compared to AM from WT mice. Furthermore, this project identified that the exposure to silica caused more LMP and greater cathepsin B release in MARCO-/- AM compared to WT AM. These results suggested that the absence of MARCO enhanced AM susceptibility to silica at the level of LMP. The mechanism of the increased LMP in the absence of MARCO was attributed to changes in cholesterol trafficking. In WT AM silica treatment resulted in increased cholesterol uptake that was greatly reduced in the absence of MARCO. Furthermore, blocking cholesterol trafficking with U186666A greatly decreased silica-induced cytotoxicity and inflammasome activation. Taken together, these results establish the important role that cholesterol plays in phagolysosomal stability and suggests a potential therapeutic site to decrease particle-induced inflammation.
This project determined the anti-inflammatory and lysosomal stabilizing potential of a lysosomotropic drug imipramine. Imipramine demonstrated anti-inflammatory effects against the inflammation caused by exposure to silica in the lungs. The results demonstrated that \textit{in vitro} pretreatment with imipramine significantly attenuated cathepsin B, a lysosomal protease, activity and release of mature IL-1β from alveolar macrophages, which indicated inhibition of NLRP3 inflammasome activation. Anti-inflammatory effect of imipramine was evident in \textit{in vitro} and \textit{in vivo} in an acute and chronic C57Bl/6 mouse exposure model. In the chronic exposure model of 42 days co-administration study protective effect of imipramine was evident in the silica-induced fibrosis in the lungs when assessed by hydroxyproline quantification. In the post-exposure study where imipramine treatment followed chronic exposure to silica, anti-inflammatory effect of imipramine was observed in attenuating lung injury following chronic exposure to silica. However, imipramine had limited effect silica-induced fibrosis in the lungs in the long-term post-administration study. Taken together, these results establish the important role that cholesterol plays in phagolysosomal stability and suggests a potential therapeutic site to decrease particle-induced inflammation. Furthermore, this project indicates that the imipramine treatment after four weeks of silica exposure did not have much effect on silica-induced chronic pathological condition. In contrast, the results establish that the concomitant or pretreatment with imipramine was effective in attenuating silica-induced lung pathology, suggesting that the early acute phase of silica-induced inflammation was the critical point where intervention could be effective. The results from this project give a prospective direction to other particulates-induced inflammation and establish that LMP is an important target for novel therapeutics for the treatment of inflammatory diseases linked to lysosomal instability. These studies can form the basis for future development of potential agent to attenuate acute and chronic inflammation due to inflammasome activation and lysosomal permeabilization.
References in Chapter 5


2. Appelqvist, H., Waster, P., Kagedal, K., Ollinger, K; The lysosome: from waste bag to potential 


4. Funk, R.S., Krise, J.P.; Cationic amphiphilic drugs cause a marked expansion of apparent lysosomal 
   volume: implications for an intracellular distribution-based drug interaction, Mol Pharmaceutics, 
   2012, 9, 1384-1395.

5. Hamilton, R.F.Jr.; Thakur, S.A.; Mayfair, J.K.; Holian, A. MARCO mediates silica uptake and 

6. Hornung, V., Bauernfeind, F; Halle, A; Samstad, E.O., Kono, H; Rock, K.L; Fitzgerald, K.A.; Latz, 
   E.; Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal 

7. Johansson, A.C.; Appelqvist, H.; Nilsson, C.; Kagedal, K.; Roberg, K.; Ollinger, K.; Regulation of 

8. Hentze, H; Lin, X.Y; Choi, M.S.K; Porter, A.G; Critical role for cathepsin B in mediating caspase-1- 
   dependent interleukin-18 maturation and caspase-1-independent necrosis triggered by the microbial 


10. Kaufmann, A.M., Krise, J.P.; Lysosomal sequestration of amine-containing drugs: analysis and 


