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**ROLE OF SCAVENGER RECEPTOR MARCO IN PARTICLE  
UPTAKE AND LUNG INFLAMMATION**

By

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Bachelor of Pharmacy, University of Mumbai, Mumbai, India, 2001

Dissertation

presented in partial fulfillment of the requirements  
for the degree of

Doctor of Philosophy

in Toxicology

The University of Montana  
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Abstract Title:

Role of scavenger receptor MARCO in particle uptake and lung inflammation

Chairperson: Andrij Holian, PhD

Alveolar macrophages (AM) form the first line of defense against chronic inflammation caused by occupational exposure to environmental particulates such as crystalline silica (CSiO<sub>2</sub>). The chronic inflammatory process triggered by CSiO<sub>2</sub> is known to culminate into a fibrotic response called silicosis in the human lungs. Previous studies have indicated the role of membrane glycoproteins called scavenger receptors in binding of environmental particles. The scavenger receptors are classified into different classes (A-H) based on their structure and function. Class A scavenger receptors are critical in uptake of variety of ligands such as bacteria, acetylated lipoproteins and are typically found on macrophages, dendritic and epithelial cells. One of the members of this family is Macrophage receptor with collagenous structure (MARCO). Recent studies have focused on analyzing the interaction between MARCO and inorganic particles such as CSiO<sub>2</sub> and titanium dioxide (TiO<sub>2</sub>). Both *in vivo* and *in vitro* binding studies have identified MARCO as a key receptor in CSiO<sub>2</sub> uptake and subsequent cytotoxicity in AM from C57Bl/6 mice. Further *in vitro* studies using a transfected cell line revealed that the 100 amino acid residues long cysteine-rich (SRCR) domain at the C-terminal end of MARCO is required for binding of inorganic particles such as CSiO<sub>2</sub>, TiO<sub>2</sub> and amorphous silica (ASiO<sub>2</sub>). Moreover, individual particles bind to SRCR domain of MARCO with unique differences and have varying requirements with respect to need for divalent cations. Our studies demonstrate that physiological absence of MARCO in C57Bl/6 mice leads to a more robust inflammatory response following CSiO<sub>2</sub> exposure as compared to wild-type mice. The results suggest that diminished clearance of CSiO<sub>2</sub> particles from the MARCO<sup>-/-</sup> lungs exacerbates the lung inflammation. These findings demonstrate that the involvement of different regions of SRCR domain may distinguish downstream events following particle binding. Taken together, these data establish the role of MARCO in uptake of various inorganic particles and elucidate the protective role of MARCO in CSiO<sub>2</sub>-induced lung inflammation.

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## LIST OF KEY ABBREVIATIONS

SR: Scavenger receptors

MARCO: Macrophage receptor with collagenous structure

CSiO<sub>2</sub>: Crystalline silica

ASiO<sub>2</sub>: Amorphous silica

TiO<sub>2</sub>: Titanium dioxide

AM: Alveolar macrophages

IM: Interstitial macrophages

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# INTRODUCTION

## 1.1. SILICOSIS

Silica is one of the most common minerals found in the earth's crust (Rosenman *et al.*, 2003). It is found in sand, rocks such as granite, sandstone and in metal quarries. Exposure to silica occurs during occupations such as construction, mining, sandblasting, grinding, drilling etc. Silica is present in nature in two forms: crystalline and non-crystalline (amorphous). Both differ significantly in the pulmonary injury they cause when inhaled. Crystalline silica (CSiO<sub>2</sub>) is a fibrogenic agent capable of inducing progressive inflammation, fibroblast proliferation and collagen deposition resulting in the development of pulmonary fibrosis known as silicosis (Craighead *et al.*, 1988; Green and Vallyathan, 1996). The clinical symptoms of silicosis cannot be seen until many years after CSiO<sub>2</sub> exposure. Early symptoms include, shortness of breath during exercise, chronic dry cough, fatigue and bluish skin, while diminished lung and vital capacity are later symptoms. 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 CSiO<sub>2</sub> particles sequestered in the middle of the nodule with macrophages and fibroblasts present on the periphery. Eventually the nodules coalesce leading to destruction and scarring of normal lung tissue.

Currently, no treatment exists for silicosis. Most treatments used are aimed at relieving the pain and discomfort due to the symptoms and do not reverse or inhibit disease progression. Patients are administered oxygen to help increase blood oxygenation

and bronchodilators to aid breathing. In addition, immunosuppressive drugs such as corticosteroids may be used in an attempt to suppress the progressive inflammation that is characteristic of the development of silicosis. At present, the only life-saving therapy is a lung transplant from a healthy donor.

## **1.2. OTHER SILICA-INDUCED DISEASES**

Exposure to CSiO<sub>2</sub> may also result in or contribute to other pulmonary diseases including chronic obstructive pulmonary disease (COPD) and increased susceptibility to tuberculosis (Craighead *et al.*, 1988; Hnizdo *et al.*, 1994). The American thoracic society defines COPD as “disease state characterized by the presence of airflow obstruction due to chronic bronchitis or emphysema” (1995). The current paradigm is that CSiO<sub>2</sub> causes chronic lung inflammation in small airways, a characteristic feature of bronchitis and destruction of lung parenchyma or emphysema, together causing COPD (Hnizdo, 1992; Hnizdo and Vallyathan, 2003). Although the mechanistic details are unknown, there are several potential mechanisms by which CSiO<sub>2</sub> may initiate cell injury leading to COPD. These include cytotoxicity and secretion of proinflammatory factors such as cytokines, chemokines and growth factors (Hamilton *et al.*, 2008; Vanhee *et al.*, 1995). With regard to increased susceptibility to tuberculosis, CSiO<sub>2</sub>-induced decrease in cell-mediated immunity and adverse effects on sensitivity of antigen presenting cells (APCs) (macrophages and dendritic cells) are known to be major contributors (Beamer and Holian, 2008; Watanabe *et al.*, 1987).

Increasing evidence from epidemiological and animal studies has implicated CSiO<sub>2</sub> exposure as a potential risk factor in development of autoimmune diseases such as systemic sclerosis, rheumatoid arthritis and systemic lupus erythematosus (Brown *et al.*,

2003; Haustein and Anderegg, 1998). The etiology of CSiO<sub>2</sub>-induced autoimmune diseases is unknown. However, it has been hypothesized that CSiO<sub>2</sub>-induced apoptosis exacerbates autoimmune responses by exposing particular autoantigens, expressed on surface of dying cells, to the immune system (Pfau *et al.*, 2004a). Moreover, CSiO<sub>2</sub> exposure may lead to impaired clearance of this dead cell debris due to overwhelmed clearance mechanisms in the lung or adverse effects on macrophage function. The impaired clearance mechanism may be another potential risk factor for development of autoimmune diseases (Cohen *et al.*, 2002). Also, CSiO<sub>2</sub> is known to act on adaptive immunity by serving as an adjuvant for stimulating T cells and causes decreases in the relative number and function of regulatory T cells (Brown *et al.*, 2004; Wu *et al.*, 2006).

Finally, CSiO<sub>2</sub> causes a variety of respiratory and systemic autoimmune diseases. Different rates and volumes of respiration, personal characteristics, genetic susceptibility, and compromised host defenses may all play a role in determining why some people may develop one or the other CSiO<sub>2</sub> associated diseases.

### **1.3. INHALATION TOXICITY FOLLOWING OCCUPATIONAL EXPOSURE TO ASiO<sub>2</sub> AND TiO<sub>2</sub>**

In contrast, exposure to amorphous silica (ASiO<sub>2</sub>) has been shown to cause only self-limited reversible pulmonary inflammation (Warheit *et al.*, 1995; Wilson *et al.*, 1979). Epidemiological studies demonstrated that workers with long-term exposure to amorphous silica (ASiO<sub>2</sub>) did not show evidence of silicosis or COPD (Choudat *et al.*, 1990; Wilson *et al.*, 1979). Another inorganic particle, titanium dioxide (TiO<sub>2</sub>) that has been widely used in many industrial applications, as well as in medical and dental prosthesis triggers similar biological events as ASiO<sub>2</sub>. While a few studies have shown

that TiO<sub>2</sub> exposure in murine models leads to a transient inflammatory response in the lung, the exposure does not progress to fibrosis (Lardot et al., 1998; Lindenschmidt et al., 1990). Both these particles are non-toxic and are not reported to be associated with silicosis, COPD or autoimmune diseases.

### ***1.3.1. Unique Bioactivity of CSiO<sub>2</sub>***

All three inorganic particles, CSiO<sub>2</sub>, ASiO<sub>2</sub> and TiO<sub>2</sub> are recognized by the macrophages and induce inflammation by releasing cytokines and growth factors (Driscoll *et al.*, 1990; Lindenschmidt *et al.*, 1990). However, only CSiO<sub>2</sub> exposure leads to persistent inflammation eventually leading to fibrosis while, ASiO<sub>2</sub> or TiO<sub>2</sub> exposure causes reversible inflammatory response. This contrasting observation raises an important question as to why certain inorganic particles (e.g., CSiO<sub>2</sub>) induce a fibrotic response in the lung while other inorganic particles (e.g. ASiO<sub>2</sub> and TiO<sub>2</sub>) do not. In this regard, the CSiO<sub>2</sub>-induced persistent toxicity towards AM as compared to the non-toxic nature of TiO<sub>2</sub> and ASiO<sub>2</sub>, has been speculated to be an important factor (Thakur *et al.*, 2008). Another long-standing paradigm in the field is that rate and extent of particle clearance from the lung is an important step in development of silicosis. In contrast to CSiO<sub>2</sub>, numerous studies have found ASiO<sub>2</sub> and TiO<sub>2</sub> to be cleared faster and more efficiently from the lungs of exposed animals (Arts *et al.*, 2007; Oghiso *et al.*, 1992). Also, the physicochemical properties of particles have been speculated to be an important factor in both cytotoxicity and clearance from the lung (Shi *et al.*, 1989; Thakur *et al.*, 2008). The negative surface charge of the particle as well as the distribution (order) of surface charge may play an important role in the unique biological activity and fibrogenic potential of these particles. It can be further speculated that the apparent paradox in the fibrotic

outcome in response to these inorganic particles may, at least in part, be related to differences in binding of these particles to cellular receptors and the signaling events triggered by these particle-receptor interactions.

#### **1.4. PULMONARY MACROPHAGES**

Macrophages form 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). Macrophages are derived from peripheral blood monocytes, which in turn are derived from bone marrow pluripotent stem cells. Macrophages are abundant in every tissue in the body and display marked heterogeneity in phenotype specific to tissue, possibly due to local interaction with other cell types in the tissue (Gordon, 2007).

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). Although 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 release immunosuppressive factors such as IL-10, IL-4, IL-13 and TGF $\beta$  (Barbarin *et al.*, 2004; Hancock *et al.*, 1998). On the other hand, immunoenhancing AM release proinflammatory factors such as TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in



response to triggers such as CSiO<sub>2</sub>. Also, 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 murine as well as human AM subpopulations, perturbation of such balance may have pronounced effect on 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). Also, AM are reported to have higher phagocytic ability as compared with the IM. Futhermore AM were effective in producing cytokines such as TNF- $\alpha$  and IFN- $\gamma$  while IM produced higher levels of IL-1 $\beta$  and IL-6 and show increased MHC-II expression along with

greater antigen presenting activity (Franke-Ullmann *et al.*, 1996). Little data exists regarding the role of IM in particle-induced diseases, when compared with AM. Nonetheless, both AM and IM play important roles in CSiO<sub>2</sub>-induced pulmonary fibrosis. Alveolar macrophages (AM) are known to play a role in phagocytosis and clearance of CSiO<sub>2</sub> along with their well-established role in the production of cytokines and fibrogenic factors (Lehnert *et al.*, 1989). While IM are responsible for trapping the CSiO<sub>2</sub> in the interstitium and stimulating interstitial lymphocytes and fibroblasts to mount an immune response and promote collagen deposition (Adamson *et al.*, 1991).

#### ***1.4.1. Pulmonary macrophages in silicosis***

The lung has direct contact with the environment and is constantly exposed to a variety of pathogens and harmful particles. Considering the surface area of the lung and the volume of potentially harmful air inspired on a daily basis, it is remarkable that so little 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 > 1.0 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 CSiO<sub>2</sub> <1.0 µm are deposited in the alveolar regions of the lung and are believed to have higher fibrogenic potential.

##### **1.4.1.1. Role of pulmonary macrophages in clearance of CSiO<sub>2</sub> particles**

The cells that are important in initial interaction between these fine particles and the lung are alveolar macrophages (AM). Alveolar macrophages are attracted to the site of particle deposition by particle-activated complement-dependent chemotactic factors on

the alveolar surface (Warheit *et al.*, 1988). Once recognized and phagocytosed by AM, CSiO<sub>2</sub> particles are cleared out of the lung by the mucociliary pathway or they are translocated to the interstitium and lymphatic system. Successful clearance of particles from the deep lung by AM results in nominal release of proinflammatory factors and minimal cell injury and inflammation (Brody *et al.*, 1982). Anything that interferes with this chain of events can lead to prolonged interaction of CSiO<sub>2</sub> with pulmonary epithelial cells and other immune cells resulting in increased cell injury and translocation of CSiO<sub>2</sub> to the interstitium. For example, in the case of heavy and continued exposure, as in a coalmine, CSiO<sub>2</sub> is not cleared from the lungs due to overwhelmed clearance mechanisms. High concentrations of particles in the lung leads to a phenomenon known as “particle overload” resulting in translocation of particle laden AM to extrapulmonary 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). Alternatively, the CSiO<sub>2</sub> is phagocytosed by interstitial macrophages and the activated interstitial macrophages secrete proinflammatory cytokine 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).

#### **1.4.1.2. CSiO<sub>2</sub>-induced apoptosis of AM and release of inflammatory cytokines**

The current paradigm in the field is that, upon inhalation of CSiO<sub>2</sub>, AM engulf particles and make an effort to clear CSiO<sub>2</sub> particles from the lungs. As described previously, under conditions of continued exposure, overwhelmed clearance mechanisms

do not remove the particles from the lung. Not only does the uncleared CSiO<sub>2</sub> migrate to the interstitium, but the particles also interact with the resident AM for prolonged periods of time. Some of those AM get activated and secrete proinflammatory cytokines while a fraction of AM undergo apoptosis due to pronounced cytotoxic effects of CSiO<sub>2</sub> (Huaux, 2007; Iyer and Holian, 1997). Some studies have suggested that the immunosuppressive AM population, described earlier, specifically undergoes apoptosis (Iyer and Holian, 1997). This apoptotic material and free CSiO<sub>2</sub> may then be re-engulfed by other AM that then release mediators like oxygen radicals, proteases and proinflammatory cytokines or undergo apoptosis (Beamer and Holian, 2005b; Rimal *et al.*, 2005). Repeated cycles of engulfment, apoptosis and release of inflammatory mediators may prolong inflammation and contribute to silicosis (Rimal *et al.*, 2005; Srivastava *et al.*, 2002). In contrast, TiO<sub>2</sub>, neither induces macrophage apoptosis nor pulmonary fibrosis (Thibodeau *et al.*, 2003). Therefore, the fibrogenic potential of a particle is thought to correlate with its ability to induce AM apoptosis (Iyer *et al.*, 1996a; Rimal *et al.*, 2005).

Some of CSiO<sub>2</sub>-exposed AM secrete inflammatory factors as cytokines, proteases and reactive oxygen species. Other activated AM interact with immune and non-immune cells and contribute in the process of recruiting neutrophils, dendritic cells and lymphocytes. Among the cytokines released by AM, TNF- $\alpha$  and IL-1 $\beta$  have been reported to play a pivotal role in development of silicosis (Shi *et al.*, 1998). Most compelling is the evidence demonstrating anti-TNF- $\alpha$  antibodies or soluble TNF receptors and IL-1 $\beta$  antagonists attenuate CSiO<sub>2</sub>-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- $\alpha$  and IL-1 $\beta$  including

lymphocyte proliferation, oxidative bursts and degranulation of inflammatory cells (Herseth *et al.*, 2008; Shi *et al.*, 1998). Furthermore, analysis of TNF- $\alpha$  and IL-1 $\beta$  levels in the lavage fluid from CSiO<sub>2</sub>-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). Increased levels of transforming growth factor (TGF- $\beta$ ) are found in CSiO<sub>2</sub>-induced granuloma. It is mainly produced by AM and epithelial cells and has multiple role in fibrogenesis such as chemoattractant for neutrophils and mitogenic stimulus for fibroblasts (Jagirdar *et al.*, 1996).

## **1.5. SCAVENGER RECEPTORS**

Although ample evidence supports the notion that upon CSiO<sub>2</sub> exposure AM play central role in particle-induced immune effects, the molecular details of the initial interaction of particles and macrophages are not well-studied. AM express a myriad of pattern recognition receptors such as Toll like receptors, C-type lectin and  $\beta$ 2 integrin and scavenger receptors (SR) on their surface to recognize and bind environmental pathogens and particles (Platt and Gordon, 2001). 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 SR family display broad ligand-binding properties overlapping between members of the family.

Mainly expressed on macrophages and dendritic cells, SR are also found on non-immune cells like epithelial cells, endothelial cells and hepatocytes (Murphy *et al.*, 2005). The SR are divided into eight different classes (A-H) based on structure and function (Classes A-H) (Murphy *et al.*, 2005). Two members of Class A family of scavenger

receptors, namely, scavenger receptor A (SRA I/II) and macrophage receptor with collagenous structure (MARCO) have been implicated in environmental particle binding (Hamilton *et al.*, 2006; Iyer *et al.*, 1996b). Therefore, this project focused on understanding the contribution of SRA I/II and mainly MARCO in CSiO<sub>2</sub> binding.

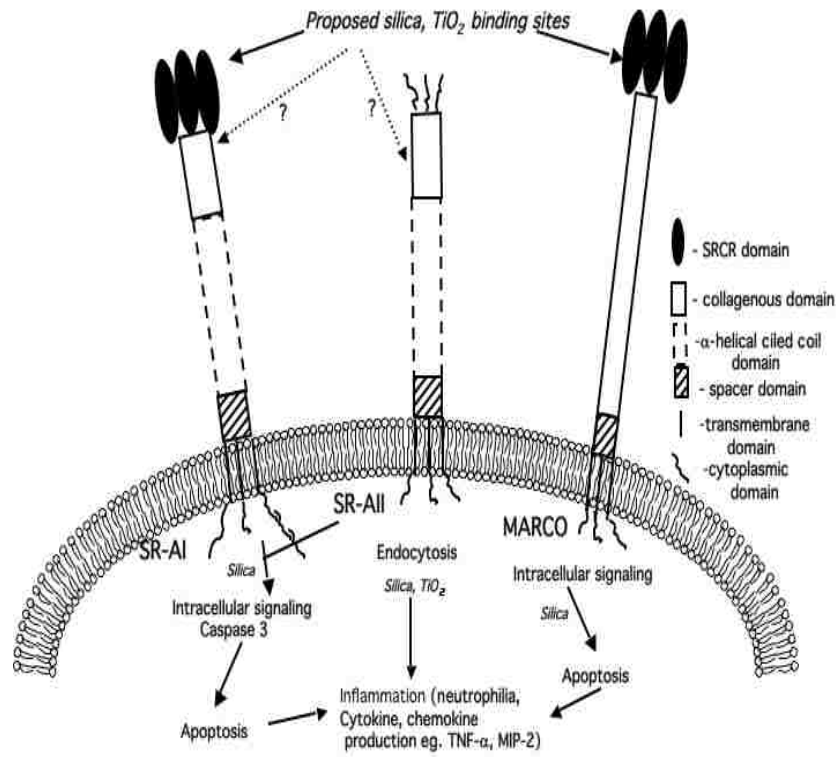
### ***1.5.1. Class A scavenger receptor family members***

The Class A scavenger receptor (SR) are expressed predominantly on macrophages and dendritic cells, mast cells, epithelial cells and endothelial cells (Brown *et al.*, 2007; Murphy *et al.*, 2005). Five members have been identified to date namely: SRA (I, II, III), MARCO, CSR1 (Cellular Stress Response 1), SRCL (SR with C-type lectin) (Moore and Freeman, 2006) and SCARA5 (Class A SR 5) (Jiang *et al.*, 2006). Similar to other classes of SR, the class A family of SR binds a wide variety of ligands including acetylated proteins, polyribonucleotides, polysaccharides, environmental particles, and play a significant role in host-defense. Examination of the SR ligands illustrates that they are polyanionic in nature, although many polyanions do not bind the SR (Platt and Gordon, 1998). The ligand binding capacity of Class A SR is broad enough to question their biological specificity. However, closer examination suggests that specificity of their binding is likely to be determined not only by the negative charge on the ligand, but also by many other factors. These may include ligand structure, surface charge distribution on the ligand, the relative affinity between the SR for a particular ligand and availability and expression of various SR.

A prototypic example of Class A SR family, SR-A I/II comprises types I and II and non functional intracellular, type III, polypeptides (Murphy *et al.*, 2005). SRA I/II is trimeric protein and each molecule is composed of six regions: a relatively short N-

terminal cytoplasmic domain, a transmembrane domain and its extracellular domain is comprised of a spacer,  $\alpha$ -helical coiled-coil, collagenous and a cysteine rich C-terminal domain (Figure 1) (Matsumoto et al., 1990). Despite the extensive similarity between the structures of SR polypeptides, some of the polypeptides lack one or more of the above mentioned domains. Some of the central structural features of SR include their trimeric nature, a collagenous domain of varying length in the extracellular region and a short N-terminal cytoplasmic domain approximately 40-60 amino acid long (Figure 1).

Furthermore, three members of Class A SR, including SRA I, MARCO and SCARA5 belong to a family of a large group of receptors called SR Cysteine Rich (SRCR) super family that have an evolutionarily conserved, SRCR domain of approximately 100-110 amino acid residues (Jiang et al., 2006; Sarrias et al., 2004). A large number of cell surface proteins with diverse functions possess the SRCR domain as an integral part of their structure. However, at present, a common function for the SRCR domain has not been determined. The known functions of the SRCR domain include ligand binding and is also implicated in immune defense mechanisms (Sarrias et al., 2004). Further studies need to be conducted to determine the role of SRCR domain in determining the specificity of ligand binding (Figure 1). With regard to this study,



**Figure 1: Schematic of Class A scavenger receptors involved in  $CSiO_2$  binding.**

The functional domains and proposed particle binding sites are indicated for SRA I, SRA II and MARCO.



because the ligand binding SRCR domain is found in both SRA I and MARCO receptors, studies were conducted to determine the role of SRCR domain in particle binding.

### ***1.5.2. Role of Scavenger Receptor Class A in binding and signaling by environmental particles***

During the 1990's, Resnick *et al.* suggested that crocidolite asbestos, an environmental particle which causes asbestosis and mesothelioma, bound efficiently to recombinant SR (SR-A I and II) (Resnick *et al.*, 1993). The binding of crocidolite asbestos to these receptors was efficiently inhibited by the non-specific SR ligands such as polyinosinic acid (poly I) and polyguanylic acid (poly G) (Resnick *et al.*, 1993). Additional studies identified other particles (*viz.*, TiO<sub>2</sub>, CSiO<sub>2</sub>, iron oxide and diesel exhaust particles) as possible ligands for SR (Iyer *et al.*, 1996a; Palecanda *et al.*, 1999b). Taken together, these studies suggested a role for the Class A family of scavenger receptors SRA (I/II) and MARCO in particle recognition and uptake.

#### **1.5.2.1. SRA I/II**

Scavenger receptor A (SRA-I/II) is expressed primarily on macrophages and has been extensively studied in the context of atherosclerosis and was initially known as a macrophage receptor for oxidatively modified lipoprotein (Dhaliwal and Steinbrecher, 1999). The first direct evidence that SRA-I/II plays a role in environmental particle induced signaling emerged when Chao *et al.* (2001) reported that CSiO<sub>2</sub>-induced caspase activation and apoptosis in a murine cell line, which was inhibited by 2F8, a monoclonal antibody to SRA I/II. In contrast to CSiO<sub>2</sub>, titanium dioxide (TiO<sub>2</sub>)-treated CHO cells expressing murine SRA I/II did not undergo apoptosis (Hamilton *et al.*, 2000). These

studies highlight the contrasting apoptotic outcome resulting from exposure to two chemically similar particles. The binding site of CSiO<sub>2</sub> on SRA I/II has not been specifically identified. However, considering the negative surface charge of CSiO<sub>2</sub>, the positively charged amino acids in the SRCR domain of SRA I are speculated to be responsible for particle binding (Figure 1). Though the SRA II polypeptide lacks the SRCR domain, a group of positively charged lysines are present in the collagenous domain of SRA II which have been found to be important for ligand binding (Figure 1) (Haberland *et al.*, 1984). Further studies focusing on identifying the amino acids responsible for CSiO<sub>2</sub> and TiO<sub>2</sub> binding would contribute to better understanding of the process of particle recognition.

Recent *in vivo* studies have provided further support for the role of SR-A I/II on AM in the innate immune response against inhaled environmental particles. SRA I/II<sup>-/-</sup> mice showed an augmented inflammatory response to CSiO<sub>2</sub> and TiO<sub>2</sub>, which included increased levels of proinflammatory cytokines like TNF- $\alpha$ , and mRNA levels of chemokine CXCL3 and significantly increased neutrophilia (Arredouani *et al.*, 2006; Beamer and Holian, 2005b). The exact mechanistic details of the role of SRA I/II in CSiO<sub>2</sub> induced apoptosis and cytokine signaling is as yet unknown (Figure 1). While these results identified the role of SRA I/II in CSiO<sub>2</sub> binding and subsequent cytotoxicity, the studies did not examine the relative contribution of SRA I/II in CSiO<sub>2</sub> binding in presence of another Class A scavenger receptor, MARCO.

#### **1.5.2.2. MARCO**

Macrophage receptor with collagenous structure (MARCO) has significant structural similarity with SRA I except that it lacks the  $\alpha$ -helical coiled-coil domain in

the extracellular region (Figure 1) (Elomaa et al., 1995). MARCO is expressed mainly on AM, macrophages in the marginal zone of spleen and lymph nodes and dendritic cells. 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 TiO<sub>2</sub> (Arredouani *et al.*, 2006; Elomaa *et al.*, 1998).

Structurally, MARCO receptor is a 210 kDA integral trimeric transmembrane protein, in which each of the three subunits (54 kDA) are composed of short 50 amino acid NH<sub>2</sub>-terminal intracellular domain, a small transmembrane domain that spans the cell membrane and an extracellular portion composed of a 75 amino acid spacer domain, a 270 amino acid collagenous domain and the 100 amino acid C-terminal cysteine rich domain (SRCR) domain (Figure 2) (Elomaa *et al.*, 1998). The SRCR domain of MARCO has been found to play a role in ligand binding and cell adhesion (Brannstrom *et al.*, 2002b). Recently, a positively charged arginine based “RXR” motif in the SRCR domain of MARCO has been reported to mediate the binding of gram positive and gram negative bacteria (Brannstrom *et al.*, 2002b). This study proposes to analyze the role of the “RXR” motif in binding of negatively charged environmental particles. Additionally, to account for the full complexity of binding behavior of MARCO, understanding the role of all other structural and physical factors, of the particles, such as size and surface charge distribution is of importance.

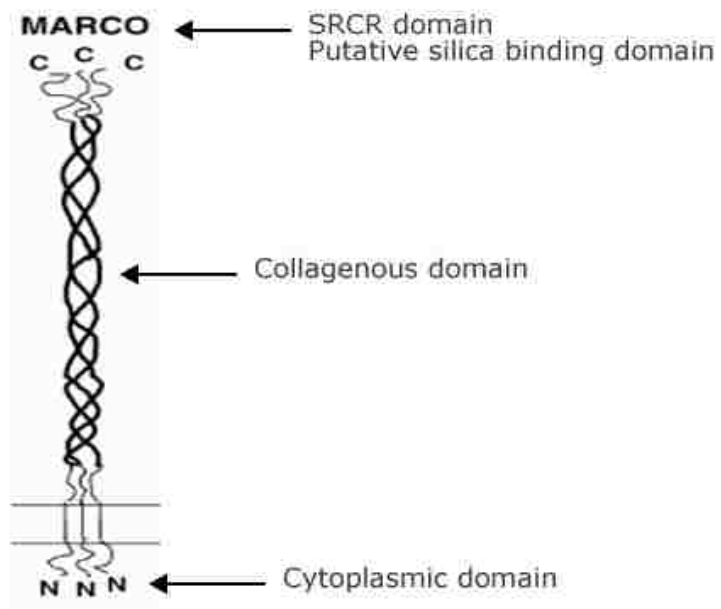


Figure 2: Schematic representation of structure of MARCO

(modified from J. E. Murphy et. al. Atherosclerosis 2005, 1-14)

Mechanistically, not much is known with respect to the signaling events triggered following particle interaction with MARCO (Figure 1). However, similar to SRA I/II<sup>-/-</sup> mice, MARCO<sup>-/-</sup> mice showed a dramatic increase in polymorphonuclear leukocyte trafficking into the lungs, increased levels of TNF- $\alpha$ , and mRNA levels of chemokine CXCL3 following TiO<sub>2</sub> exposure (Arredouani et al., 2006). Although the direct link between MARCO and enhanced neutrophilia remains uncertain, it has been attributed to increased expression of chemokine CXCL3, a potent neutrophil chemoattractant (Arredouani et al., 2006). These results suggested a protective role of MARCO in the lungs against the CSiO<sub>2</sub> exposure.

## **1.6. SPECIFIC AIMS**

This project is mainly focused on identifying the role of scavenger receptor MARCO in CSiO<sub>2</sub> binding and cytotoxicity. Alveolar macrophages (AM) from both SRA I/II and MARCO single and double knockout mice were used to elucidate the relative contribution of SRA I/II and MARCO in CSiO<sub>2</sub> binding and cytotoxicity. Further *in vitro* studies using human MARCO transfected Chinese hamster ovary (CHO) cells examined the differential binding of toxic CSiO<sub>2</sub> and non-toxic TiO<sub>2</sub> and ASiO<sub>2</sub> to MARCO. Understanding the differences in binding patterns of these inorganic particles may help explain the varying signaling events triggered by inorganic particles, despite binding to a common receptor, MARCO. Additionally, by using MARCO<sup>-/-</sup> mice, the role of MARCO in CSiO<sub>2</sub> induced acute and chronic inflammation was analyzed. The results from this study will help understand the molecular details involved in the initial macrophage-particle interaction and examine the implications of this interaction on subsequent disease

process. The role of MARCO in CSiO<sub>2</sub> induced disease pathology was examined using following three specific aims:

**Specific Aim I:**

To characterize the role of MARCO in CSiO<sub>2</sub> binding and alveolar macrophage cytotoxicity (Chapter 1 and Appendix A)

**Specific Aim II:**

To identify the particle-binding domain of MARCO and to investigate whether the difference in cytotoxicity of various particles may be related to their differential binding to MARCO (Chapter 2)

**Specific Aim III:**

To characterize the role of MARCO in CSiO<sub>2</sub> induced pulmonary inflammation (Chapter 3)

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## CHAPTER ONE

### MARCO MEDIATES CRYSTALLINE SILICA UPTAKE AND TOXICITY IN ALVEOLAR MACROPHAGES FROM C57BL/6 MICE

#### ABSTRACT

Scavenger receptors (SR) expressed on the surface of the macrophage, appear to be responsible for crystalline silica (CSiO<sub>2</sub>) uptake and cell death signaling in the macrophages. The purpose of this study was to investigate relative contribution of macrophage receptor with collagenous structure (MARCO) and scavenger receptor A (SRA I/II) in CSiO<sub>2</sub> binding, using various SR null mice. The findings demonstrated that MARCO was the critical SR involved in CSiO<sub>2</sub> uptake and cytotoxicity in the primary alveolar macrophages (AM) from C57BL/6 mice, as there was no particle uptake or cell death in the absence of this SR. The level of MARCO expression on AM changed significantly with the absence of other SR, and CSiO<sub>2</sub> uptake was proportional to cell surface MARCO expression. In addition, CSiO<sub>2</sub> uptake and cytotoxicity were completely blocked by an anti-mouse MARCO antibody. Transfection of Chinese hamster ovary cells with human MARCO further supported these conclusions, as CSiO<sub>2</sub> particles bound to and initiated apoptosis in the MARCO-transfected cells. Inability of the MARCO<sup>-/-</sup> AM in binding CSiO<sub>2</sub> resulted in decreased ability of AM in modifying the antigen-induced T cell stimulation, a characteristic of MARCO<sup>+</sup> AM. Taken together, these results indicate MARCO is the primary AM receptor interacting with CSiO<sub>2</sub>, depending on mouse strain and level of constitutive expression.

## 2.1. INTRODUCTION

Inhaled crystalline silica (CSiO<sub>2</sub>) particles are known initiator of several human pathologies including silicosis, autoimmune disorders and possible even lung cancer (Peretz *et al.*, 2006). The earliest contact with inhaled crystalline silica (CSiO<sub>2</sub>) occurs when alveolar macrophages (AM) recognize and engulf CSiO<sub>2</sub> particles. This can lead to cell death in a fraction of the exposed cells, which may be the initial step in disease process. Scavenger receptors (SR) on the surface of the AM are one possible mechanism for uptake and cell death signaling in the AM (Gough and Gordon, 2000; Kobzik, 1995)

Scavenger receptors (SR) are transmembrane glycoproteins found on macrophages, endothelial cells, and smooth muscle cells that bind to a number of ligands including gram-negative bacteria, apoptotic cells, oxidized low-density lipoproteins and polyinosinic acid to name just a few (Gough and Gordon, 2000). The SR are divided into several different classes based on structure and function and they all bind a broad range of ligands with polyanionic surface characteristics (Murphy *et al.*, 2005). The exact function of SR is still being determined, but they are believed to be an important feature of the innate immune response and they are generally upregulated in response to bacterial infection (Hampton *et al.*, 1991; Haworth *et al.*, 1997). Due to the promiscuous nature of these receptors, multiple functions have been suggested including, but not limited to, endocytosis followed by receptor recycling, transcytosis, intracellular signaling, and uptake of particles with polyanionic surface characteristics (Murphy *et al.*, 2005; Palecanda and Kobzik, 2001; Platt and Gordon, 2001).

Class A SR include MARCO, three splice variants of SR-A (I, II, III), and SRCL and recently discovered SCARA 5 (Arredouani and Kobzik, 2004; Murphy *et al.*, 2005; Nakamura *et al.*, 2001). SRA I/II has been shown to be involved with the uptake of



titanium dioxide (TiO<sub>2</sub>), CSiO<sub>2</sub>, diesel particles, and latex beads in AM (Kobzik, 1995). In addition, CSiO<sub>2</sub>-induced cytotoxicity was demonstrated following the transfection of Chinese hamster ovary (CHO) cells with SRA I/II (Hamilton *et al.*, 2000). In this instance, TiO<sub>2</sub> was not toxic to the transfected CHO cells indicating a specific toxic response to CSiO<sub>2</sub> particles, which was inhibited by known antagonists of SRA I/II (Hamilton *et al.*, 2000).

Similarly, MARCO has been identified as the main binding receptor for unopsonized particles and bacteria on human AM using MARCO-transfected COS cells (Arredouani *et al.*, 2005; Palecanda *et al.*, 1999a), and the presence of MARCO may be the frontline defense for pneumococcal pneumonia, and clearance of inhaled particles (Arredouani *et al.*, 2004). Despite the similarity in structure and function between MARCO and SRA I/II, there is evidence that their regulation and signaling properties may be different. This is based on work with MARCO and SRA I/II null mice, where significant differences in peritoneal macrophage IL-12 production were observed following LPS and IFN $\gamma$  stimulation (Jozefowski *et al.*, 2005). In additional findings, MARCO expression was increased by Th1 polarizing factors and decreased by Th2 polarizing agents. This pattern was reversed for SRA I/II expression indicating that these proteins are differentially regulated (Jozefowski *et al.*, 2005). Mouse strain differences in MARCO and SRA I/II expression have also been observed, with MARCO being the constitutively expressed SR in the C57BL/6 strain (Palecanda and Kobzik, 2001; Su *et al.*, 2001). In humans, the expression of MARCO under normal circumstances is relatively low compared to SRA I/II, but it can be induced by a variety of stimulants including the presence of bacteria (Jozefowski *et al.*, 2005).

The study was conducted to determine the relative contribution of the two SR (MARCO and SRA I/II) on the uptake and toxicity of CSiO<sub>2</sub> particles by the murine AM, using single and double null mice on the C57BL/6 background. The primary hypothesis of this work is that MARCO mediates CSiO<sub>2</sub> uptake and toxicity in the C57BL/6 mouse due to the fact that MARCO is constitutively expressed SR on the AM.

## **2.2. MATERIALS AND METHODS**

### **Mice**

Breeding pairs of C57BL/6 mice were originally purchased from The Jackson Laboratory (Bar harbor, ME, USA); while breeding pairs of SRAI/II<sup>-/-</sup> and MARCO<sup>-/-</sup> mice on C57BL/6 background were kindly provided by Dr. Lester Kobzik (Harvard School of Public Health, Boston, MA). Genotyping was carried out as described previously (Dahl *et al.*, 2007). All mice were maintained in the University of Montana specific pathogen-free (SPF) laboratory animal facility. The mice (age; 7-10 weeks) were maintained on an ovalbumin-free diet and given deionized water *ad libitum*. The University of Montana Institutional Animal Care and Use Committee (IACUC) approved all animal procedures.

### **Particles**

Crystalline silica (CSiO<sub>2</sub>) (min-U-sil-5), 1 – 5 µm in diameter from Pennsylvania Sand Glass Corp. (Pittsburgh, PA) was acid-washed in 1M HCl at 100°C. The CSiO<sub>2</sub> was then washed in sterile water three times and dried in an oven at 200°C to remove all water. A stock suspension of 2.5 mg/ml in PBS was generated before each experiment.

The stock suspension was dispersed by sonic disruption for 1 min prior to use. Titanium dioxide (TiO<sub>2</sub>) was purchased from Fisher Scientific (Cat# T315-500 Houston, TX), and used unprocessed.

### **Alveolar Macrophage Isolation and Culture**

Mice were euthanized by a lethal injection of Euthasol, the lungs were removed and then lavaged with five 1.0 ml aliquots of cold phosphate buffered saline (PBS). Pooled cells were pelleted at 400 x g for 5 min and cell pellet was resuspended in 1 ml of RPMI 1640 culture media (Mediatech Inc., Herdon, VA) supplemented with 10 % fetal calf serum (Mediatech), antibiotics and antimycotics (Gibco, Carlsbad, CA). Total lavage cells were enumerated using a Z1 Coulter Particle Counter (Beckman Coulter, Hialeah, FL). The cells were adjusted to 10<sup>6</sup> per ml and added to 0.65 ml sterile polypropylene tubes at 500 µl/tube. The CSiO<sub>2</sub> suspensions were added and the cells were cultured in a tumbling suspension culture for 4 h at 37°C in a water-jacketed CO<sub>2</sub> (5%) incubator (ThermoForma, Mariette, OH). The azide free SR antibodies included anti-mouse MARCO (R and D Systems, Minneapolis, MN) and anti mouse SRA I/II (Serotec, Raleigh, NC).

### **Alveolar Macrophage Viability Assay**

Isolated alveolar macrophages were cultured in suspension (10<sup>6</sup> cells/ml) with varying concentrations of CSiO<sub>2</sub> for 4 h at 37°C. At the end of this period, culture supernatant was removed and mixed with 0.4% trypan blue solution (Sigma, St. Louis, MO). The resulting mixture was added to a hemocytometer and the cells were examined by light microscopy. Random cells were counted per sample, and cells that appeared to

contain blue dye were considered dead. Data was expressed as percent of cells excluding trypan blue dye (percent living cells).

### **Alveolar Macrophage Apoptosis Assay**

Apoptosis was determined using the Cell Death ELISA (Roche Biochemicals, Indianapolis, IN), and was performed according to the manufacture's protocol. Briefly, isolated alveolar macrophages were cultured in suspension ( $10^6$  cells/ml) with varying concentrations of CSiO<sub>2</sub> for 4 h at 37° C. At the end of this period 100 µl of the culture supernatant ( $10^5$  cells) was removed, washed with PBS, and the resulting cell pellet was lysed with the buffer provided in the kit. The lysate was assayed in ELISA format for histone-bound DNA fragments. The optical density (OD) is read at 405 nm and the background was subtracted out of the final results. Data is expressed as the average OD  $\pm$  SEM.

### **Determination of MARCO Expression on Alveolar Macrophages**

Immediately following the AM isolation described above, the cells were exposed to Fc block (1:50) for 30 min at room temp. Anti-F480 PE (Caltag, Burlingame, CA), and anti-CD11c APC (BD Pharmingen), were then added to the cells at a 1:50 dilution along with anti-mouse MARCO FITC (Serotec, Raleigh, NC) at a 1:5 dilution, allowed to incubate for 30 min at room temperature with agitation 2-3 times. Finally, AM were washed twice with PBS and resuspended in 0.5 ml PBS and analyzed immediately on a FACS Aria flow cytometer using FACS Diva software (Becton Dickinson). Flow cytometric methods detected cells that were positive for MARCO FITC, while gating on

alveolar macrophages that were dual positive for the markers CD11c and F4-80. Data are expressed as the MARCO positive percentage of CD11c/ F4-80 positive cells.

### **Quantification of CSiO<sub>2</sub> Uptake for Alveolar Macrophage**

Use of flow cytometric side scatter properties to quantify cell/particle binding and uptake is described elsewhere (Stringer *et al.*, 1995). In this study, following CSiO<sub>2</sub> exposure for 1.5 hours, the cells were exposed to FcBlock (1:50) for 30 min at room temperature. Anti-F480-PE (Caltag, Burlingame, CA) and Anti-CD11c APC (BD Pharmingen) were added to the cells at a 1:50 dilution and allowed to incubate for 30 min at room temperature, with agitation 2-3 times. Finally, AM were washed twice with PBS and resuspended in 0.5 ml PBS and analyzed immediately on a FACs Aria flow cytometer using FACS Diva software (Becton Dickinson). Flow cytometry analysis detected CSiO<sub>2</sub> uptake into the AM by assaying for changes in forward and side scatter properties while gating on AM which were dual positive for the markers CD11c and F4-80 (Crowell *et al.*, 1992). Data are expressed as average side scatter intensity with arbitrary numerical units.

### **Transfection of Chinese Hamster Ovary Cells**

Chinese Hamster Ovary cells (CHO K1) were cultured in Ham's F-12 (Cellgro) with 10% FBS plus 100 IU/ml penicillin and 100 µg/ml streptomycin (Mediatech). CHO cells were plated at 1 x 10<sup>6</sup> cells/well in a 6-well cell culture dish overnight and transiently transfected with 4 µg of pcDNA3.1 and hMARCO cDNA using Lipofectamine 2000 (Invitrogen Life Technologies) according to manufacturer's protocol. The cells were harvested and used after 36-40 h. The cells were harvested and

resuspended in PBS containing 0.1 % sodium-azide and 2 % BSA were preincubated with or without primary mAb PLK-1 (7 µg/ml) for 15 min on ice. The cells were then treated with TiO<sub>2</sub> (25 µg/ml), or CSiO<sub>2</sub> (150 µg/ml) and rotated at 37°C for 30 min, placed on ice and analyzed by flow cytometry. Binding of particles was measured using increase in mean side scatter as a marker of increase in granularity of cells indicating uptake of CSiO<sub>2</sub> and TiO<sub>2</sub> particles.

### **Human MARCO surface expression on transiently transfected CHO cells**

For immunofluorescence, 10<sup>6</sup> cells suspended in PBS containing 0.1 % sodium azide and 2 % BSA were incubated with mAb PLK-1 (7 µg/ml) and Alexa 488 conjugated AcLDL (2.5 µg/ml) at 37°C for 30 min. The cells were washed twice with PBS containing containing 0.1 % Na-azide and 2 % BSA. After washing the antibody PLK-1 was detected using Alexa-488 conjugated goat anti-mouse IgG by flow cytometry. The human MARCO cDNA in pcDNA 3.1 and primary mAb PLK-1 (human MARCO) was provided by Dr. L. Kobzik (Harvard School of Public Health, Boston, MA).

### **Apoptosis detection in MARCO-transfected CHO Cells**

Briefly, 5 x 10<sup>5</sup> CHO cells were seeded in 6-well plates and incubated for 24 h. The cells were then transiently transfected as described above. The transfected cells were allowed to recover for 24 h. Cells were then treated with 50 µg/cm<sup>2</sup> of CSiO<sub>2</sub> for 7 h at 37°C. The cells were scraped and centrifuged at 1500 x g for 10 min at 4°C. The pellets were washed once with 2 ml of PBS, then permeabilized in 1 ml of ice-cold 80% ethanol and left on ice for 1 h. Cells were centrifuged at 1000 x g at 4 °C for 10 min, washed once with 3 ml of ice-cold PBS, and stained with 50 µg/ml propidium iodide in PBS with

0.1% Triton X-100, 0.1 mM EDTA and 100 µg/ml RNase overnight at 4 °C. Apoptosis measurement (the sub-G0/G1 population) was determined on a FACs Aria Flow cytometer using FACs DIVA software (BD Biosciences).

### **Lymphocyte Isolation and Culture**

OT-II transgenic mice on a C57BL/6 background were euthanized by a lethal injection of Euthasol and their spleens were removed and placed in Hank's buffered saline (Invitrogen) with 2 % heat-inactivated fetal bovine serum (Mediatech) on ice. The spleens were ground up between two sterile frosted glass slides and filtered through sterile gauze into 50-ml centrifuge tubes. The cell suspension was centrifuged 200 x g for 10 min. The nucleated cell fraction was counted by lysing the red blood cells with Zapaglobin reagent followed by the Z1 Coulter particle counter (Beckman Coulter, Hialeah, FL). The cell suspension was adjusted to  $5 \times 10^7$  nucleated cells per ml in the media described above and the Spin Sep™ murine T cell enrichment (Stem Cell Biotechnology, Vancouver, BC, Canada) was performed according to the manufacturer's protocol. The resulting cell recovery was >96% CD 3 positive by FACS analysis. The T cells were suspended in RPMI 1640 culture media (Mediatech Inc) supplemented with antibiotics and antimycotics (Invitrogen) at  $2 \times 10^6$  cells/ml.

### **Antigen-presenting Cell Assay**

Following a 1 h AM suspension culture  $\pm$  CSiO<sub>2</sub> (100 µg/ml) or TiO<sub>2</sub> (50 µg/ml), the AM were plated in 96-well tissue culture plates (Costar, Corning, NY) at  $5 \times 10^4$  cells/well and allowed to adhere. Ovalbumin (Sigma) antigen (10 mg/ml), or anti-CD3 antibody (5 µg/ml) was added. This mixture was incubated 2 h at 37°C in a water-

jacketed CO<sub>2</sub> (5%) incubator (ThermoForma, Mariette, OH). The isolated transgenic T cells were added to each well at  $2 \times 10^5$  cells/well. This mixture was incubated 48 h at 37°C. After 2 days the 96-well plate was centrifuged at 1000 x g for 3 min and the supernatant was retrieved and stored frozen at -20°C until it could be assayed for cytokine levels.

### **Cytokine Assays**

Culture supernatants were assayed for cytokines with commercially available kits according to the manufacturer's protocol. Interferon  $\gamma$  (IFN $\gamma$ ) measurements were determined by using Duo-set kits (R and D Systems). Samples were diluted 1:100. Interleukin-13 (IL-13) measurements were determined using Duo-set kits (R & D Systems). Samples were diluted 1:2. Colorimetric analysis was performed with the Spectra Max 340 plate reader (GE Healthcare) at 450 nm. Data are expressed as picograms/ml of retrieved culture supernatant.

### **Statistical Analyses**

All one-factor experimental designs were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's comparison to a single control group. All two-factor experimental designs were analyzed by two-way ANOVA followed by selected Bonferroni's post hoc pair-wise mean comparisons. Pearson's correlation was used to determine significant associations between factors. Sample size varied between 3 and 8 experimental replications depending on the experiment and desired statistical power. Statistical significance was established as a one-tailed probability of type I error



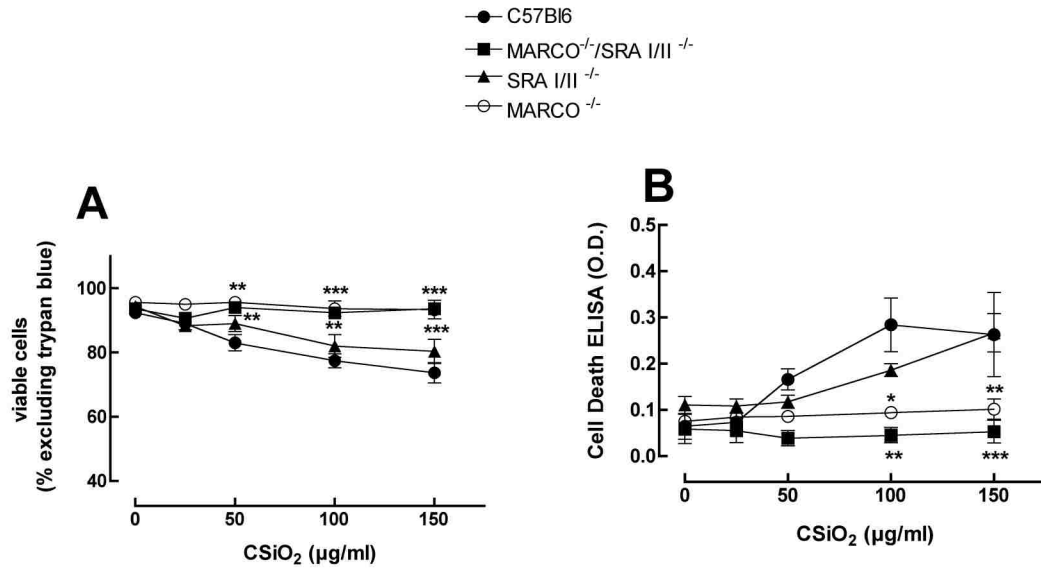
occurring at less than 5%. Analysis and graphics were performed on Prism 4.0 software (GraphPad, San Diego, CA).

## 2.3. RESULTS

### **Cytotoxicity of CSiO<sub>2</sub> in scavenger receptor knockout mice on C57Bl/6 background**

To evaluate the activity of different SR in crystalline silica (CSiO<sub>2</sub>)-induced cytotoxicity of AM, a variety of SR single and double null mice on the C57Bl/6 background were used. The effect of CSiO<sub>2</sub> on alveolar macrophages (AM) cell viability in a 4 h suspension culture showed that AM from the SRA I/II<sup>-/-</sup> was not significantly different from wild-type (WT) with CSiO<sub>2</sub> causing a concentration-dependent loss in cell viability in both WT and SRA I/II<sup>-/-</sup> (Figure 1A). In contrast, the AM from MARCO<sup>-/-</sup> and MARCO<sup>-/-</sup>/SRA I/II<sup>-/-</sup> mice showed no loss of viability for any concentration of CSiO<sub>2</sub> tested. These differences were statistically significant when compared to the corresponding WT values at 50, 100, and 150 μg/ml CSiO<sub>2</sub> (Figure 3A).

Further the effect of CSiO<sub>2</sub> on AM cell apoptosis in a 4 h suspension culture indicate that AM from the SRA I/II<sup>-/-</sup> were not significantly different than WT AM. CSiO<sub>2</sub> caused a concentration-dependent increase in cell apoptosis in both WT and SRA I/II<sup>-/-</sup> AM (Figure 3B). Consistent with the observation above, the AM from MARCO<sup>-/-</sup> and MARCO<sup>-/-</sup>/SRA I/II<sup>-/-</sup> mice showed no apoptosis for any concentration of CSiO<sub>2</sub> tested. These differences were also statistically significant when compared to the corresponding WT values at 100, and 150 μg/ml CSiO<sub>2</sub> (Figure 3B). Taken together, these results suggest that MARCO was important and SRA I/II was not important for AM cytotoxicity on C57Bl/6 mice.

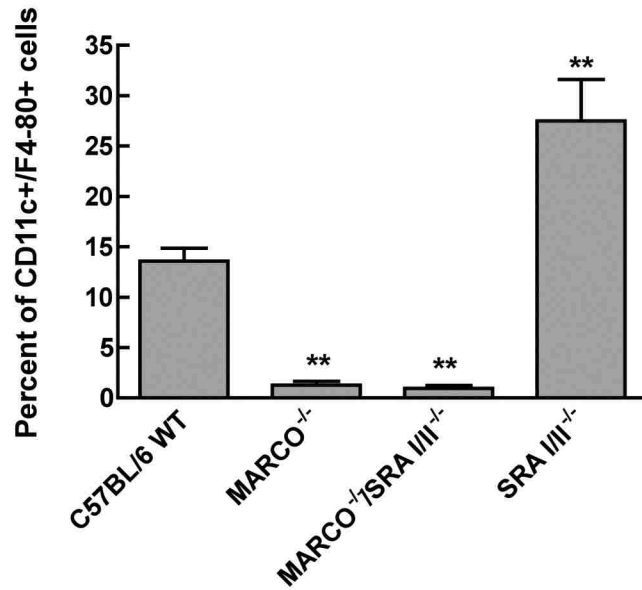


**Figure 3: Role of MARCO in CSiO<sub>2</sub> induced AM cytotoxicity.**

(A) Alveolar macrophage viability. Mean  $\pm$  SEM percent of cells excluding trypan blue dye following a 4 h suspension culture with CSiO<sub>2</sub> particles (0, 25, 50, 100, and 150 µg/ml). (B) Alveolar macrophage apoptosis. Mean  $\pm$  SEM optical density at 405 nm following a 4 h suspension culture with CSiO<sub>2</sub> particles (0, 25, 50, 100, and 150 µg/ml). For both graphs: ● indicates alveolar macrophages (AM) from C57 wild-type; ■ AM from MARCO<sup>-/-</sup>/SRA I/II<sup>-/-</sup>; ○ AM from SRA I/II<sup>-/-</sup>; ▲ AM from MARCO<sup>-/-</sup>. \* indicates P < 0.05, \*\* indicates P < 0.01 and \*\*\* indicates P < 0.001 compared to corresponding wild-type control by Bonferroni's post hoc test. Sample size n = 3.

### **Relative MARCO Expression in Scavenger Receptor Knockout C57Bl/6 Mice**

Based on the results in Figure 3, MARCO surface expression was determined for AM from both WT and KO mice (Figure 4). All of the AM from null mice had a significantly different amount of MARCO surface expression from WT AM. The AM from MARCO<sup>-/-</sup> and MARCO<sup>-/-</sup>/SRA I/II<sup>-/-</sup> had significantly less MARCO expression than WT AM, with the MARCO<sup>-/-</sup> having no expression above background. The AM from SRA I/II<sup>-/-</sup> mice had significantly increased levels of MARCO expression suggesting a compensatory role of MARCO in SRA I/II<sup>-/-</sup> mice in C57Bl/6 model (Figure 4).



**Figure 4: Relative expression of MARCO on AM from SR knockout mice**

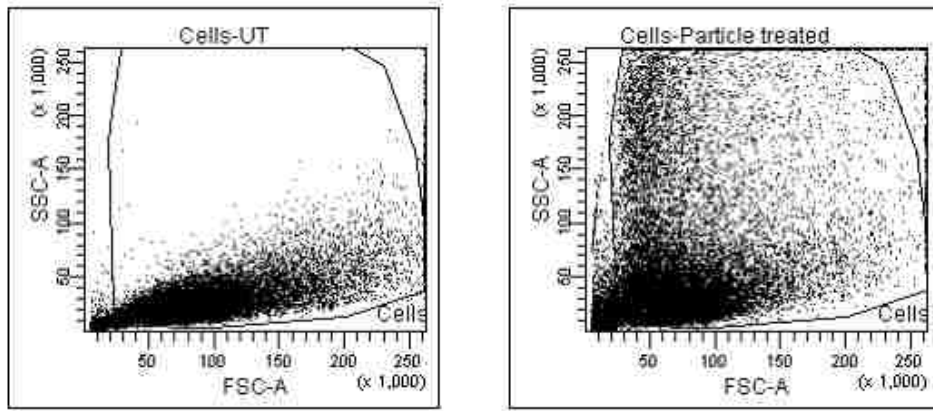
Mean  $\pm$  SEM percent of CD11c/F4-80 positive cells expressing MARCO on the cell surface immediately following cell isolation. \*\* indicates  $P < 0.01$  compared to corresponding wild-type control by Dunnett's comparison to a single control group.

Sample size  $n = 3$ .

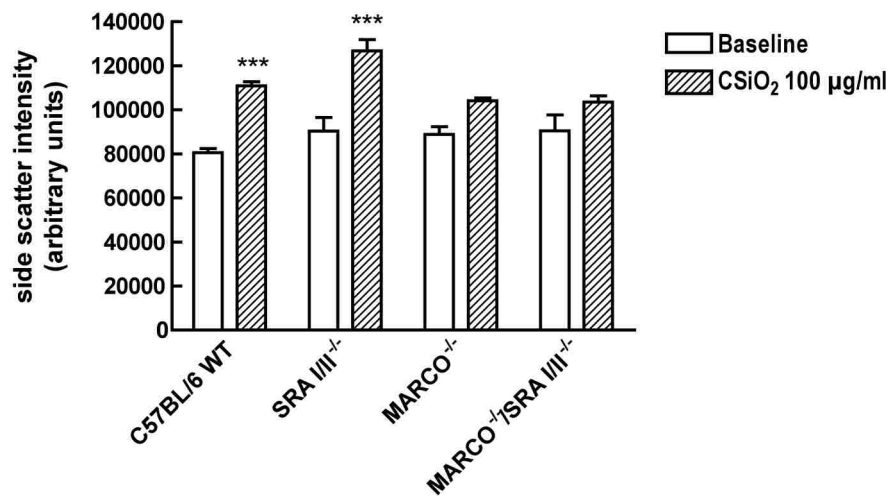
### **Relative CSiO<sub>2</sub> Uptake by AM from Scavenger Receptor Knockout C57Bl/6 Mice**

To further evaluate the role of each SR in AM cytotoxicity, CSiO<sub>2</sub> uptake was determined for all WT and null AM by measuring increase in side scatter properties following CSiO<sub>2</sub> exposure, using flow cytometry. Binding of non fluorescent environmental particles such as CSiO<sub>2</sub> and titanium dioxide (TiO<sub>2</sub>) by the cells leads to increase in side scatter properties. This increase is used as a measure for particle uptake by cells (Figure 5A). Following a 1.5 h suspension culture at 37° C, CSiO<sub>2</sub> (100 μg/ml) produced a significant increase in side scatter compared to baseline (no particle control) in AM from WT mice (Figure 5B). Likewise, AM from SRA I/II<sup>-/-</sup> mice had significant increases in side scatter with CSiO<sub>2</sub> exposure compared to the corresponding baseline control. The AM from MARCO<sup>-/-</sup> and MARCO<sup>-/-</sup>/SRA I/II<sup>-/-</sup> mice showed a slight non-significant increase in side scatter with CSiO<sub>2</sub> exposure. While, AM from SRA I/II<sup>-/-</sup> showed the highest side scatter in response to CSiO<sub>2</sub> (Figure 5B). The baselines side scatter (CSiO<sub>2</sub>) across groups was not significantly different. There was no detectable CSiO<sub>2</sub>-induced apoptosis or necrosis (potentially confounding the side scatter data) at the end of the 90-min culture. Therefore, these results suggest that MARCO plays an important role in CSiO<sub>2</sub> uptake and cytotoxicity in the C57Bl/6 mice.

**A**



**B**



**Figure 5: Relative CSiO<sub>2</sub> uptake by AM from SR knockout mice.**

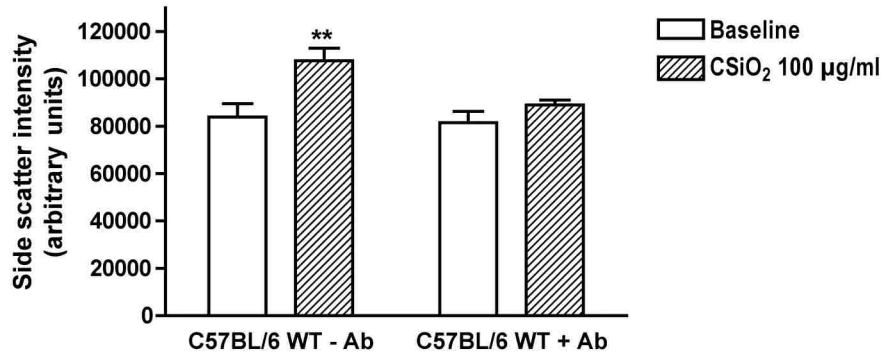
(A) Representative flow cytometry scatter plots showing the increase in side scatter properties of cells following particle (TiO<sub>2</sub>) treatment, similar results are obtained following CSiO<sub>2</sub> treatment. (B) Mean ± SEM side scatter intensity following 1.5 hr suspension culture with or without silica particles (100 µg/ml). Open bars indicate no CSiO<sub>2</sub>. Hatched bars indicate CSiO<sub>2</sub> exposure. \* indicates P < 0.05, and \*\*\* indicates P < 0.001 compared to corresponding ‘no CSiO<sub>2</sub>’ control by Bonferroni’s post hoc test.

Sample size n =3

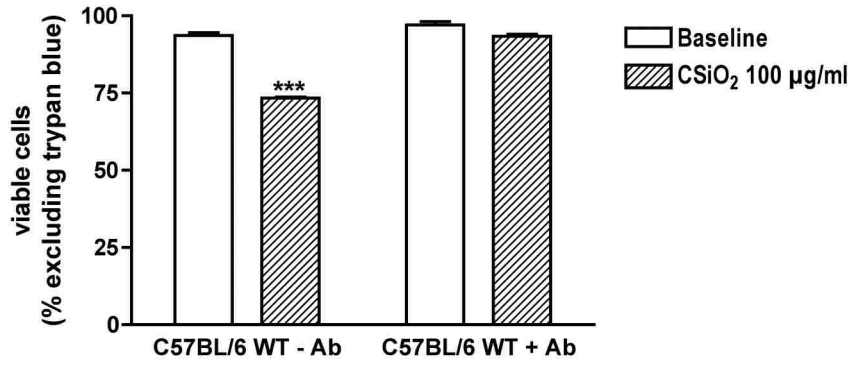
### **MARCO antibody blocks CSiO<sub>2</sub> uptake and cytotoxicity**

Because MARCO appeared to be the best candidate for CSiO<sub>2</sub> binding and toxicity, AM from WT mice were used with an anti-mouse MARCO antibody. Briefly, anti-mouse MARCO (azide free, R & D Systems) at 5 µg/ml was pre-incubated with the AM for 30 min prior to CSiO<sub>2</sub> exposure followed by treatment with and without CSiO<sub>2</sub> (100 µg/ml) for additional 4 h. These results show that significant increases in side scatter with CSiO<sub>2</sub> exposure are completely reversed in the presence of MARCO antibody (Figure 6A). Likewise, the significant loss of viability seen in the presence of CSiO<sub>2</sub> (100 µg/ml) is also completely inhibited by MARCO antibody (Figure 6B). Consistent with the viability data, CSiO<sub>2</sub> -induced apoptosis is significantly attenuated in the presence of MARCO antibody (Figure 6C). Because MARCO antibody is completely effective in preventing CSiO<sub>2</sub> uptake and toxicity, it can be concluded that SRA I/II does not contribute to CSiO<sub>2</sub> recognition in C57Bl/6 AM.

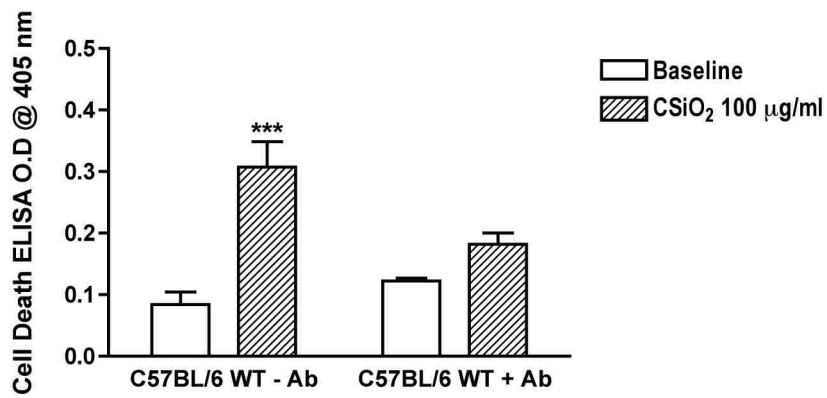
A



B



C





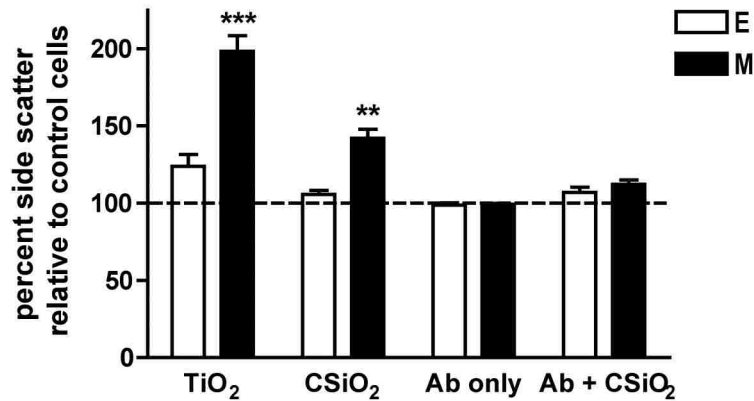
**Figure 6: Anti-mouse MARCO blocks CSiO<sub>2</sub> uptake and toxicity in C57Bl/6 AM.**

(A) Mean  $\pm$  SEM side scatter intensity following 1.5 h suspension culture with or without CSiO<sub>2</sub> particles (100  $\mu$ g/ml). (B) Mean  $\pm$  SEM percent of cells excluding trypan blue dye following a 4-hr suspension culture with or without CSiO<sub>2</sub> particles (100  $\mu$ g/ml). (C) Mean  $\pm$  SEM optical density at 405 nm following a 4 h suspension culture with or without CSiO<sub>2</sub> particles (100  $\mu$ g/ml). Open bars indicate no CSiO<sub>2</sub>. Hatched bars indicates CSiO<sub>2</sub> exposure. \*\* indicates  $P < 0.01$  and \*\*\* indicates  $P < 0.001$  compared to corresponding 'no CSiO<sub>2</sub>' control by Bonferroni's post hoc test. Sample size  $n = 3$ .

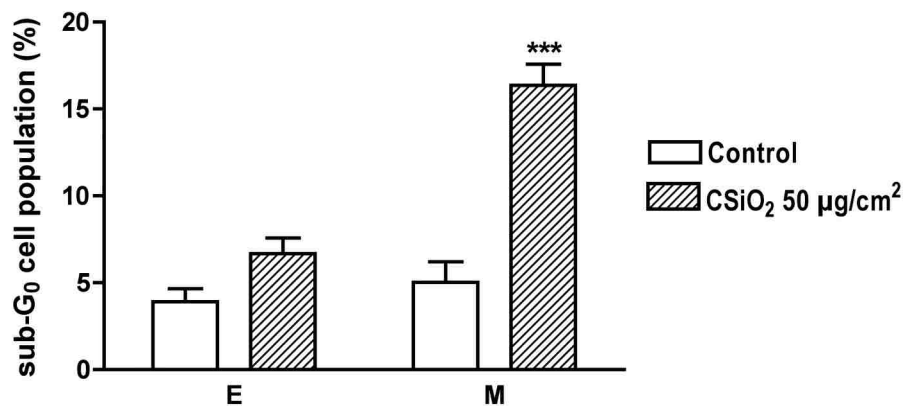
### **The effect of Human MARCO Transfection on Chinese Hamster Ovary Cells**

To further evaluate the role of MARCO in CSiO<sub>2</sub> binding, Chinese hamster ovary (CHO) cells were transfected with human MARCO (hMARCO) as described in Methods. The resulting transfected cells were exposed to CSiO<sub>2</sub> (150 µg/ml) and TiO<sub>2</sub> (25 µg/ml) for 30 min in suspension culture at 37°C. Following particle treatment, there was an increase in side scatter of hMARCO transfected CHO cells (Figure 7). Treatment with TiO<sub>2</sub> particles led to a more dramatic increase in side scatter as compared to CSiO<sub>2</sub> particles, indicating that there were differences in the binding pattern of the two particles (Figure 7). Changes in the side scatter properties following treatment of the CHO cells CSiO<sub>2</sub> and TiO<sub>2</sub> are shown in the representative scatter plots in Figure 7. The increase in side scatter properties of cells indicate that both TiO<sub>2</sub> and CSiO<sub>2</sub> binding was significantly increased in hMARCO transfected CHO cells (Figure 8). While the empty vector transfected did not bind or showed little particle binding. Furthermore, with regard to CSiO<sub>2</sub>, binding was blocked by the pre-addition of anti-hMARCO (PLK-1) antibody (Figure 8A). Apoptosis assay, measuring increases in sub G0/G1 cell population showed that the hMARCO transfected CHO cells show significant amount of apoptosis in comparison with mock-transfected cells (Figure 8B). This effect was partially inhibited by pre-incubation with anti-human MARCO antibody (PLK-1) (data not shown).

A



B



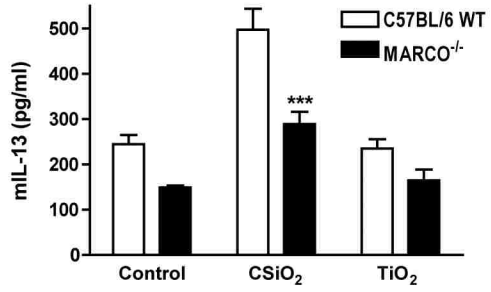
**Figure 7: The effect of human MARCO transfection on CHO cells.**

(A) Mean  $\pm$  SEM percent side scatter relative to unstimulated control cells following 30 min particle exposure in suspension culture. Open bars indicate empty vector control transfection (E). Solid bars indicate human MARCO (M) transfection. (B) Mean  $\pm$  SEM, Percent sub G<sub>0</sub>/G<sub>1</sub> cells. Open bars indicate no CSiO<sub>2</sub>. Hatched bars indicate CSiO<sub>2</sub> exposure. \*\* indicates  $P < 0.01$  and \*\*\* indicates  $P < 0.001$  compared to corresponding 'empty vector (E)' control by Bonferroni's post hoc test. Sample size  $n = 3-5$

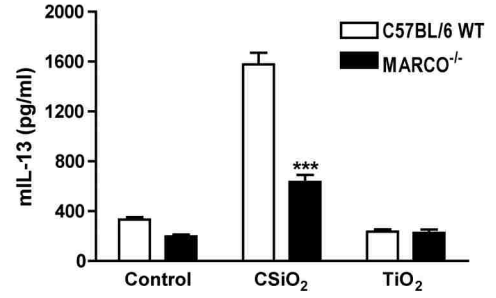
**Alveolar macrophages from MARCO<sup>-/-</sup> are resistant to CSiO<sub>2</sub>-induced APC hyper-stimulation of T cells.**

Having established the importance of MARCO in recognition, binding, and toxicity of CSiO<sub>2</sub> particles, it was necessary to test the hypothesis that CSiO<sub>2</sub> modulations to macrophage function would likewise be affected. The CSiO<sub>2</sub> particle effect on macrophage APC activity is well described elsewhere (Hamilton *et al.*, 2001; Migliaccio *et al.*, 2005). The results of the APC activity assay illustrate that lymphocyte-derived cytokines IL-13 and IFN- $\gamma$  resulting from antigen-dependent (ovalbumin-stimulated) and antigen-independent (anti-CD3-stimulated) macrophage/lymphocyte co-cultures (Figure 8, A-D). The CSiO<sub>2</sub>-induced APC hyper-response is evident in all 4 graphs (Figure 8, A-D). This effect was specific to CSiO<sub>2</sub>, as TiO<sub>2</sub> did not stimulate cytokine release significantly above control. In addition, the CSiO<sub>2</sub>-stimulated MARCO<sup>-/-</sup> AM co-cultures had significant reductions in T cell cytokine release compared with wild type regardless of the stimulant (ovalbumin or CD3 antibody). The difference in Fig. 8C failed to reach statistical significance, although it does represent a 50% reduction in IFN- $\gamma$  release relative to control. The absence of MARCO have functional consequences with regard to the behavior of AM exposed to CSiO<sub>2</sub> particles, suggesting that CSiO<sub>2</sub> binding, internalization, and/or cytotoxicity are necessary for the increase in APC activity. However, the internalization of CSiO<sub>2</sub> may be the most important factor as MARCO null AM still had a residual increase in APC activity, possibly due to a non-receptor-mediated endocytosis.

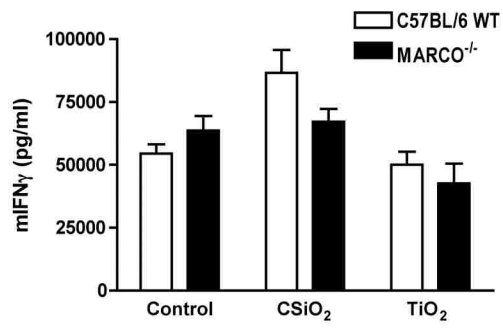
A



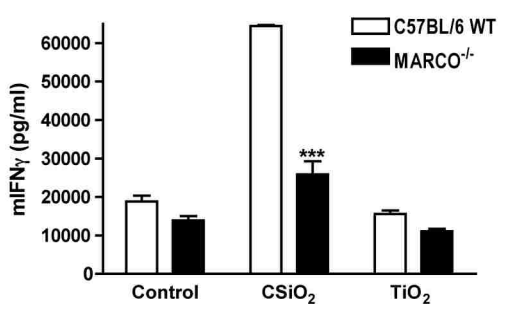
B



C



D



**Figure 8. Alveolar macrophages from MARCO<sup>-/-</sup> mice are resistant to CSiO<sub>2</sub>-induced APC hyperstimulation.**

(A) Mean ± SEM. Ovalbumin (OVA)-stimulated IL-13 release in 48 h macrophage/lymphocyte coculture. (B) Mean ± SEM. Anti-CD3-stimulated IL-13 release in 48-h macrophage/lymphocyte co-culture. (C) Mean ± SEM. ovalbumin-stimulated IFN-γ release in 48-h macrophage/lymphocyte co-culture. (D) Mean ±S.E. anti-CD3-stimulated IFN-γ release in 48-h macrophage/lymphocyte co-culture. Open bars indicate AM from C57BL/6 wild type. Black bars indicate AM from MARCO<sup>-/-</sup>. \*\*\*, indicates p <0.001 compared with corresponding wild-type control by Bonferroni's post hoc test. Sample size n =3.

## 2.4. DISCUSSION

The key finding from this study is that scavenger receptor MARCO is solely responsible for crystalline silica (CSiO<sub>2</sub>) uptake and toxicity in C57Bl/6 alveolar macrophages (AM). The importance of MARCO in this model may be attributed to the observation that it is the primary constitutively expressed SR (of the ones examined) on the C57Bl/6 AM. Several studies have reported overlapping functions between SRA I/II and MARCO, this is an example of “convergent evolution” where proteins from different genes develop a similar response pattern and function (Kobzik, 1995; Murphy *et al.*, 2005; Palecanda *et al.*, 1999a) Therefore this study examined relative contributions of SRA I/II and MARCO, however MARCO was clearly found to be the important receptor for CSiO<sub>2</sub> binding in C57Bl/6 model.

The CSiO<sub>2</sub> cytotoxicity studies (Figure 3) demonstrated that the absence of MARCO was critical to blocking the cytotoxicity of CSiO<sub>2</sub>. Only AM from the MARCO<sup>-/-</sup> and MARCO<sup>-/-</sup>/SRA I/II<sup>-/-</sup> mice were completely resistant to CSiO<sub>2</sub>-induced cytotoxicity. Interestingly, the absence of SRA I/II did not have an effect in this model. This could be explained by the possibility that SRA I/II is not constitutively expressed on C57BL6 AM. The gene deletion, in this case, would be of no consequence. Also, analysis of MARCO expression on AM from various SR null mice demonstrated that SRA I/II<sup>-/-</sup> mice have significantly enhanced MARCO expression suggesting a compensatory role of MARCO in CSiO<sub>2</sub> binding by these AM (Figure 4). However, the increased MARCO expression on SRA I/II<sup>-/-</sup> AM did not contribute to CSiO<sub>2</sub> increased cytotoxicity (Figure 1). Another possible explanation is that the polymorphism in C57BL6 SRA I/II results in a nonfunctional receptor incapable of interacting with CSiO<sub>2</sub>. There is ample evidence that SRA I/II interacts with CSiO<sub>2</sub> particles initiating apoptosis

in AM and other cell models as defined by increase in caspase 3 activity and increases in sub G0/G1 population (Chao *et al.*, 2001; Hamilton *et al.*, 2001). In addition, a recent publication shows that SRA I/II is important in binding bacteria and environmental particles in an *in vivo* murine model (Arredouani *et al.*, 2006).

The relative distribution of MARCO in the various null strains (Figure 4) suggests that the absence of SRA I/II may influence the expression level of MARCO. This might indicate a compensatory mechanism with regard to SR expression due to various overlapping functions of SR. Regardless of the strain, a relatively small percentage of AM (~ 14 %) express MARCO constitutively (Figure 4). This could be due to maturation state of the AM, with MARCO expression higher in more mature AM.

The result of the CSiO<sub>2</sub> uptake study (Figure 5B) was consistent with the results presented above on CSiO<sub>2</sub> cytotoxicity. The absence of MARCO, regardless of single or double KO model, resulted in a significant reduction for AM CSiO<sub>2</sub> uptake. However, it did not completely eliminate CSiO<sub>2</sub> uptake. There was still some CSiO<sub>2</sub> uptake in the MARCO null models, indicating that a possible alternative process for taking up CSiO<sub>2</sub> may exist on these AM cells. Nevertheless, the level of MARCO expression was highly correlated with the amount of CSiO<sub>2</sub> uptake in different knockout strains, indicating the receptor-mediated mechanism was dominant in the C57Bl/6 model. In contrast, the cytotoxicity measures were not significantly correlated to MARCO expression, regardless of which measure was used (viability or apoptosis). This may indicate that CSiO<sub>2</sub> cytotoxicity is a more complex phenomenon than the particle uptake. Not much is known about intracellular MARCO signaling, but studies on SRA I/II ligand binding reveal activation of phospholipase C- $\gamma$ 1, phosphatidylinositol 3-kinase, protein kinase C



(Hsu *et al.*, 1998), heterotrimeric G proteins (Whitman *et al.*, 2000), mitogen-activated protein kinases (Hsu *et al.*, 2001), and caspases (Chao *et al.*, 2001). Due to the similarity in structure and function between MARCO and SRA I/II, it seems reasonable to speculate that MARCO would initiate similar intracellular responses.

Studies conducted using human MARCO transfected CHO cells demonstrated that only human MARCO transfected CHO cells bound CSiO<sub>2</sub> while, the empty vector transfected CHO cells did not (Figure 7). This also demonstrates that CSiO<sub>2</sub> binding with the MARCO receptor is not an artifact of using the C57BL6 model indicating relevance to the human condition.

In conclusion, the C57BL6 AM uses MARCO for the uptake and processing of silica particles. In a percentage of the AM population, this leads to significant amount of cell death depending on the amount of CSiO<sub>2</sub> encountered by the cells. This scenario is different in other mouse strains because of different receptors responsible for the processing of CSiO<sub>2</sub>. There are probably multiple mechanisms at work in the human lung with regard to the processing of inhaled CSiO<sub>2</sub>, depending on the constitutive expression of these scavenger receptors for any given exposed individual. It may partially explain the variable susceptibilities to CSiO<sub>2</sub> exposure and subsequent difference on disease pathologies in humans.

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## CHAPTER TWO

### DIFFERENTIAL BINDING OF INORGANIC PARTICLES TO MARCO

#### ABSTRACT

Alveolar macrophages (AM) in the lung have been documented to play pivotal roles in inflammation and fibrosis (silicosis) following inhalation of crystalline silica (CSiO<sub>2</sub>). In contrast, exposure to either titanium dioxide (TiO<sub>2</sub>) or amorphous silica (ASiO<sub>2</sub>) is considered relatively benign. The scavenger receptor MARCO, expressed on AM, binds and internalizes environmental particles such as silica and TiO<sub>2</sub>. Only CSiO<sub>2</sub> is toxic to AM, while ASiO<sub>2</sub> and TiO<sub>2</sub> are not. We hypothesize that differences in induction of pathology between toxic CSiO<sub>2</sub> and non-toxic particles ASiO<sub>2</sub> and TiO<sub>2</sub> may be related to their differential binding to MARCO. In vitro studies with CHO cells transfected with human MARCO and mutants were conducted to better characterize MARCO-particulate (e.g. ASiO<sub>2</sub>, CSiO<sub>2</sub> and TiO<sub>2</sub>) interactions. Results with MARCO transfected CHO cells and MARCO specific antibody demonstrated that the scavenger receptor cysteine rich (SRCR) domain of MARCO was required for particle binding for all the tested particles. Only TiO<sub>2</sub> required the divalent cations Ca<sup>+2</sup> and/or Mg<sup>+2</sup> for binding to MARCO. Furthermore, results from competitive binding studies supported the notion that TiO<sub>2</sub> and each of the silica particles bound to different motifs in SRCR domain of MARCO. The results also suggest that particle shape and/or crystal structure may be the determinants linking particle binding to MARCO and cytotoxicity. Taken together, these results demonstrate the SRCR domain of MARCO is required for particle

binding and that involvement of different regions of SRCR domain may distinguish downstream events following particle binding.

### 3.1. INTRODUCTION

Prolonged occupational exposures (mining, construction, etc.) to inhaled crystalline silica (CSiO<sub>2</sub>) particles can lead to an irreversible and many times fatal fibrotic condition of the lungs called silicosis (Hamilton *et al.*, 2008; Ng and Chan, 1992). Currently, no effective treatment exists for silicosis, which is a significant health problem throughout the world, particularly in developing nations (Saiyed and Tiwari, 2004).

Silica is one of the most abundant minerals found on the surface of earth; it exists in crystalline and amorphous forms: both of which contribute to occupational exposure. However, of the two silica types, CSiO<sub>2</sub> is known to be the causative agent for silicosis. Although amorphous silica (ASiO<sub>2</sub>) has been reported to cause pulmonary inflammation following inhalation, it does not lead to silicosis (Merget *et al.*, 2002; Reuzel *et al.*, 1991). Similarly, another inorganic particle, titanium dioxide (TiO<sub>2</sub>) is relatively inert and is widely used in many industrial applications, as well as in medical and dental prosthesis (Driscoll *et al.*, 1991; Lardot *et al.*, 1998; Lindenschmidt *et al.*, 1990).

Inhaled particles are initially encountered by the first line of defense, alveolar macrophages in the lungs. Alveolar macrophages (AM) are cells that are primarily responsible for binding, ingestion and ultimately clearance of inhaled particulate matter (Hamilton *et al.*, 2008). When AM encounter CSiO<sub>2</sub> they have been shown to rapidly engulf the particles and undergo apoptosis (Hamilton *et al.*, 2008; Iyer *et al.*, 1996b). Consequently, it is possible that AM engulf both apoptotic bodies and free CSiO<sub>2</sub>

particles and then secrete proinflammatory cytokines or undergo apoptosis. This cycle of engulfment, apoptosis and cytokine secretion can lead to recruitment of other inflammatory cells that contribute to prolonged inflammation and development of fibrosis (Beamer and Holian, 2007; McCabe, 2003). In contrast, ASiO<sub>2</sub> and TiO<sub>2</sub> (at sizes used in this study) do not induce macrophage apoptosis, suggesting that apoptosis could play a role in initiating the fibrotic development by silica (Arts *et al.*, 2007; Iyer *et al.*, 1996b; Thibodeau *et al.*, 2003). This phenomenon is further supported by studies that indicate that the fibrogenic potential of a particle correlates with its ability to induce apoptosis in AM (Iyer *et al.*, 1996b; Rimal *et al.*, 2005).

Scavenger receptors (SR) are cell surface glycoproteins capable of binding a broad spectrum of ligands including oxidized and acetylated lipoproteins and bacterial pathogens (Murphy *et al.*, 2005). Recent studies have demonstrated SR involvement in environmental particle binding and lung inflammation (Arredouani *et al.*, 2006; Hamilton *et al.*, 2006). A member of the scavenger receptor family is MACrophage Receptor with COLLagenous structure (MARCO) expressed by macrophages, dendritic cells and certain endothelial cells. Previous studies identified MARCO as a key receptor in recognizing CSiO<sub>2</sub> and causing apoptosis in murine AM (Hamilton *et al.*, 2006). While, MARCO has also been reported to be a receptor for TiO<sub>2</sub> and does not cause apoptosis (Arredouani *et al.*, 2005). However, even though MARCO binds both CSiO<sub>2</sub> and TiO<sub>2</sub>, they show contrasting apoptotic and pathological outcomes (Thakur *et al.*, 2008).

MARCO is a 210 kDa trimeric, type II membrane protein comprised of a short intracellular and a large extracellular domain, a transmembrane domain and a C-terminal cysteine rich (SRCR) domain (Elomaa *et al.*, 1995; Kraal *et al.*, 2000). The SRCR

domain is an ancient, highly conserved domain consisting of 100 amino acid residues (Sarrias *et al.*, 2004). Proteins expressing this highly conserved motif are classified into a SRCR superfamily and have a wide range of functions often associated with the innate immune system (Rast *et al.*, 2006; Sarrias *et al.*, 2004). The SRCR domain of MARCO has been shown to be a binding site for bacteria, lipopolysaccharide and acetylated lipoproteins (Brannstrom *et al.*, 2002a; Chen *et al.*, 2006). Recently, the SRCR domain of MARCO was crystallized and was found to possess a basic cluster containing several arginines (positively charged) and a separate acidic cluster containing a bound metal ion (negatively charged). Both clusters were reported to be involved in ligand binding (Ojala *et al.*, 2007). Taking into consideration the negative surface charge of environmental particles such as silica and TiO<sub>2</sub>, it can be postulated that the positively charged arginines in the SRCR domain of MARCO might be important for binding of the particles. Although previous studies have focused on determining the role of MARCO in binding of environmental particles, the exact binding site has not been determined. Collectively, we hypothesize that the difference in induction of pathology between toxic CSiO<sub>2</sub> and the non-toxic ASiO<sub>2</sub> and TiO<sub>2</sub> may be related to differential binding to MARCO, and the signaling events triggered by these particle-MARCO interactions. The two primary goals of the present study were to identify the particle-binding domain of MARCO and to examine the parameters that influence particle binding to MARCO.



## 3.2. MATERIALS AND METHODS

### Mice

SRA I/II null mice on C57Bl/6 background were kindly provided by Dr. Lester Kobzik (Harvard school of public Health, Boston, MA). Genotyping was carried out as described previously (Dahl *et al.*, 2007). All mice were maintained in the University of Montana specific pathogen-free (SPF) laboratory animal facility. The mice were maintained on an ovalbumin-free diet and given deionizer water *ad libitum*. The University of Montana Institutional Animal Care and Use Committee (IACUC) approved all animal procedures.

### Particles

Crystalline silica (CSiO<sub>2</sub>) (Min-U-Sil-5) obtained from Pennsylvania Sand Glass Corporation (Pittsburg, PA) was acid washed in 1 M HCl at 100°C, to remove metals and microbial contamination. The CSiO<sub>2</sub> particles were then washed three times with sterile water and dried at 200°C to remove all water. Titanium dioxide (TiO<sub>2</sub>) particles were purchased from Fischer Scientific (T315-500). The DAPI conjugated amorphous silica (ASiO<sub>2</sub>) particles 1 µm in diameter were purchased from Postnova Analytics, Inc. (Salt Lake City, UT). The ASiO<sub>2</sub> particles were washed three times with sterile phosphate buffered solution (PBS) to remove the shipping medium. For all binding and cytotoxicity experiments the particles were suspended in either PBS-Azide Buffer (PAB), (0.1 % sodium azide, 1 % FBS in PBS) or Ham's F-12 media at 2.5 mg/ml. The stock suspensions were dispersed by sonic disruption for 1 min before each experiment. For all experiments CSiO<sub>2</sub> and ASiO<sub>2</sub> were used at a concentration of 150 µg/ml. Since TiO<sub>2</sub>

particles were smaller in size (100-200 nm) as compared to CSiO<sub>2</sub> and ASiO<sub>2</sub> (1-2.5 μm), the cells were treated with 25 μg/ml or 75 μg/ml in an effort to keep the number as well as the surface area of particles comparable, but sufficient for binding studies.

### **Alveolar Macrophage Isolation and Culture**

Mice were euthanized by a lethal injection of Euthasol™. The lungs were removed with the heart and then lavaged with five 1 ml aliquots of cold PBS. Pooled cells were centrifuged at 400 μg for 5 min. The lavage fluid was aspirated and discarded. The cell pellet was resuspended in 1 ml of RPMI 1640 culture media supplemented with 10 % fetal bovine serum, 100 IU penicillin, 100 μg/ml streptomycin (Mediatech Inc., Herdon, VA). Total lavage cells were enumerated using a Z1 Coulter Particle Counter (Beckman Coulter). The cells were adjusted to 10<sup>6</sup> per ml and added to 0.65 ml sterile polypropylene tubes at 500 μl/tube as described previously (Scheule *et al.*, 1992). Particulates were added and the cells were cultured in a tumbling suspension culture for 4h at 37°C in a water-jacketed CO<sub>2</sub> (5%) incubator (ThermoForma, Mariette, OH).

### **Alveolar Macrophage Viability Assay**

Isolated AM were cultured in suspension (10<sup>6</sup> cells/ml) with different concentrations of particles for 4 h at 37°C. At the end of this period, 10 μl of the culture supernatant was removed and mixed with 10 μl of 0.4% trypan blue solution (Sigma). The resulting mixture was added to a hemacytometer and the cells were examined by light microscopy. One hundred random cells were counted per sample, and cells that

appeared to contain blue dye were considered dead. Data were expressed as percent of cells excluding trypan blue dye (percent living cells).

## **Cell Culture**

Chinese Hamster Ovary (CHO) cells (American Type Culture Collection, Manassas, VA) were cultured in HAM's F-12 medium with 2 mM L-glutamine (Mediatech Inc) containing 10% heat-inactivated fetal bovine serum (FBS), 100 IU penicillin, 100 µg/ml streptomycin (Mediatech Inc.). CHO cells were transiently transfected with pcDNA 3.1 (E), full length human MARCO (M), truncated MARCO (Mt) lacking the entire SRCR domain, MARCO with only the initial 22 amino acids in the SRCR domain (M442) or MARCO mutant with only 11 amino acids (lacking the RGR motif) in the SRCR domain (M431). Scavenger Receptor A I (SRA I) was included as an expression control. All transfections were conducted using lipofectamine 2000 as per manufacturer's instructions (Invitrogen). Transfection efficiency of the full length MARCO and the various mutants was determined to be 30-40 % by staining the cells for MARCO expression using human MARCO specific antibody (PLK-1) kindly provided by Dr. Lester Kobzik (Harvard School of Public Health) and isotype control (IgG3) plus FITC conjugated secondary antibody (Southern Biotechnology Associates, Birmingham, AL). Analysis was done using FACS Aria flow cytometer using Diva Software (version 4.1.2; BD Biosciences). All the experiments were conducted 36-40 h following transient transfections.

## **In vitro binding of inorganic particles**

Transiently transfected CHO cells were harvested using trypsin and the cells were resuspended in 1 ml of PAB and counted. Cells,  $1 \times 10^6$ , were treated with or without 10  $\mu\text{g/ml}$  of monoclonal antibody against the SRCR domain of human MARCO (PLK-1) or 10  $\mu\text{g/ml}$  isotype control IgG3 on ice for 15 min. The cells were then treated with different concentrations of  $\text{CSiO}_2$ ,  $\text{TiO}_2$  and  $\text{ASiO}_2$  for 30 min at  $37^\circ\text{C}$  in tumbling suspension culture. Particle binding was then measured as an increase in mean side scatter (nonfluorescent  $\text{CSiO}_2$  and  $\text{TiO}_2$ ) as previously described (Hamilton *et al.*, 2006; Palecanda and Kobzik, 2000) and as increase in DAPI positive cells (fluorescent DAPI  $\text{ASiO}_2$  particles) by FACS Aria flow cytometer using Diva Software (version 4.1.2; BD Biosciences). The analysis of the  $\text{CSiO}_2$  and  $\text{TiO}_2$  particle binding included changes in side scatter of all cells (transfected and untransfected).

### **Cell surface expression of human MARCO variants in CHO cells**

Transiently transfected CHO cells (grown in  $10 \text{ cm}^2$  culture dishes) were washed twice with ice-cold PBS containing 1 mM  $\text{CaCl}_2$  and 1 mM  $\text{MgCl}_2$  ( $\text{Ca}^{+2}/\text{Mg}^{+2}$ ) and then incubated on ice with 0.5 mg/ml Biotin (Pierce, Rockford, IL) for 15 min. Biotin treatment was repeated again for 15 min on ice. The cells were then washed once with ice-cold PBS ( $\text{Ca}^{+2}/\text{Mg}^{+2}$ ) and lysed in cell lysis buffer. The cell lysate was then centrifuged and transferred to 1.5 ml eppendorf tubes. Forty microliters of Neutravidin beads (Pierce) were added to the cleared lysate and rotated at  $4^\circ\text{C}$  for 2h. The beads were then washed four times with the ice-cold cell lysis buffer. The protein was eluted from the beads with 2x sample buffer and denatured by heating at  $70^\circ\text{C}$  for 10 min. The 30  $\mu\text{l}$  of bead-free lysate was then fractionated by 10 % Bis-Tris NuPAGE gel (Invitrogen) and

transferred to nitrocellulose membrane. The nitrocellulose membrane was incubated with monoclonal antibody against the intracellular domain of human MARCO. The membrane was incubated with an anti-rabbit HRP-labeled secondary antibody (R & D Systems, Minneapolis, MN) and protein signals were visualized with a chemiluminescent reagent (ECL Plus Western Blotting detection system; Amersham Biosciences, Piscataway, NJ)

### **Cytotoxicity assay (YOPRO-1/PI assay)**

Transiently transfected CHO cells were harvested using trypsin and the cells were resuspended in Ham's F-12 media and counted. Cells,  $1 \times 10^6$  were treated with either CSiO<sub>2</sub>, ASiO<sub>2</sub> or TiO<sub>2</sub> for 15 min in a tumbling suspension culture at 37°C to ensure particle binding to the cells. The treated cells were then plated in 6-well plates at 37°C or 6h. After 6h the cells were trypsinized, centrifuged and resuspended in 1 ml of PBS, followed by addition of 1 µm of propidium iodide (PI) and 1 µm YOPRO-1, a DNA intercalant dye (Idziorek *et al.*, 1995) that stains only apoptotic cells (Molecular Probes, Eugene, OR) for 20 min. The percentages of apoptotic (Yopro-1-positive) and late apoptotic (Yopro-1 and PI-positive) cells were immediately determined by FACS Aria flow cytometer using FACS DIVA software (version 4.1.2; BD Biosciences).

### **Effects of CSiO<sub>2</sub> or TiO<sub>2</sub> pretreatment on ASiO<sub>2</sub> binding**

Transiently transfected CHO cells were harvested using trypsin and the cells were resuspended in 1 ml of PAB and counted. Cells,  $1 \times 10^6$  were pretreated with 50 µg/ml of CSiO<sub>2</sub> or 75 µg/ml of TiO<sub>2</sub> for 15 min prior to different concentrations of ASiO<sub>2</sub> exposure for 30 min at 37°C in tumbling suspension culture. The uptake of the ASiO<sub>2</sub> particles was

immediately measured as an increase in DAPI fluorescent cells by FACS Aria flow cytometer using Diva Software (version 4.1.2; BD Biosciences).

### **Effect of divalent cations $\text{Ca}^{+2}$ and $\text{Mg}^{+2}$ on $\text{CSiO}_2$ , $\text{ASiO}_2$ and $\text{TiO}_2$ binding**

Transiently transfected CHO cells were harvested using trypsin and the cells were resuspended in 5 ml of Dulbecco's PBS (dPBS) ( $\text{Ca}^{+2}$  and  $\text{Mg}^{+2}$  free) (Invitrogen) supplemented with 2 mM EDTA to chelate any remaining divalent cations present in the cell suspension. The cells were then washed twice with 5 ml of dPBS to remove residual EDTA. Cells,  $10^6$  per ml were suspended in dPBS and treated with  $\text{TiO}_2$  (25 or 75  $\mu\text{g/ml}$ ),  $\text{CSiO}_2$  (150  $\mu\text{g/ml}$ ) and  $\text{ASiO}_2$  (150  $\mu\text{g/ml}$ ) in presence or absence of 5 mM  $\text{CaCl}_2$  and/or 5mM  $\text{MgCl}_2$  (final concentration). The effect of divalent cations ( $\text{Ca}^{+2}$  and  $\text{Mg}^{+2}$ ) on particle binding was then measured as an increase in mean side scatter (SSc) by FACS Aria flow cytometer using Diva Software (version 4.1.2; BD Biosciences).

### **Particle size measurement and zeta potential assays.**

Particle size was measured by scanning electron microscopy (SEM) and to study the effect of the suspension media the size was analyzed using light scattering techniques by Dr. Nianqiang Wu (West Virginia University, WV). Hitachi S4700 scanning electron microscope (SEM) was used to image the particles. Prior to SEM observation, the particle suspension was dropped on the surface of a Si wafer and then dried in ambient condition. The size of  $\text{CSiO}_2$  particles was measured by Malvern Mastersizer 2000 particle size analyzer. The particles were suspended in deionized water, PAB or Ham's F-12 media, sonicated and then analyzed using Nanosizer and Microsizer instruments at

RT. The surface charge on the particles was measured by calculating the zeta potential of the particle (samples were analyzed by Colloidal Sciences Laboratory, Westampton, NJ).

### **Statistical Analysis**

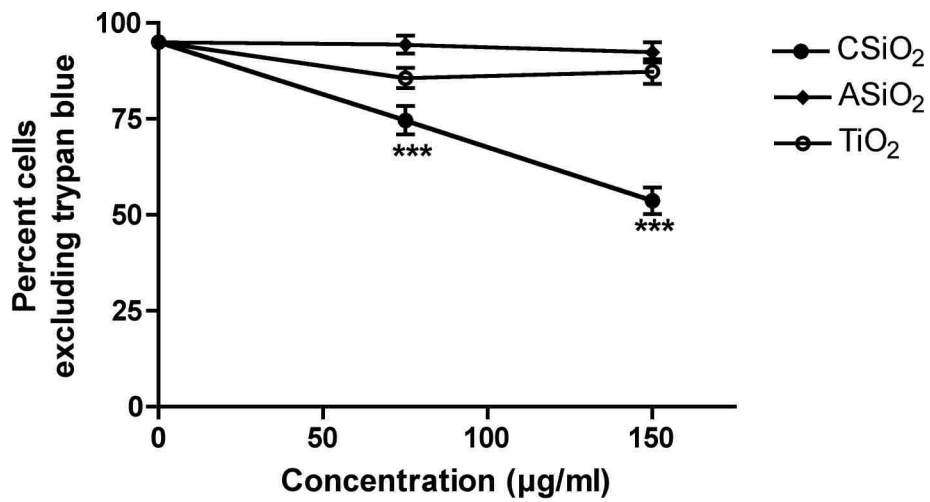
Data were analyzed using the Prism Software, version 4 (GraphPad Prism, San Diego, CA). The significance of differences between treatment groups and controls was determined using one-way ANOVA or two-way analysis of variance in conjunction with Bonferroni's post hoc analysis depending on the experiment. Data are represented as mean  $\pm$  SE. A value of  $p < 0.05$  was considered significant.

### 3.3. RESULTS

#### **CSiO<sub>2</sub>, but not ASiO<sub>2</sub> or TiO<sub>2</sub>, is toxic to AM:**

Previous reports have demonstrated that in comparison with crystalline silica (CSiO<sub>2</sub>), other inorganic particles such as amorphous silica (ASiO<sub>2</sub>) and titanium dioxide (TiO<sub>2</sub>) are not toxic (Iyer *et al.*, 1996b). To confirm previous reports of relative toxicity of CSiO<sub>2</sub>, ASiO<sub>2</sub> and TiO<sub>2</sub>, the effect of these particles on AM viability was analyzed. Primary AM from SRA I/II<sup>-/-</sup> mice were treated with different concentrations ASiO<sub>2</sub>, CSiO<sub>2</sub> and TiO<sub>2</sub> for 4 h in suspension-culture and changes in cell viability were measured using trypan blue exclusion assay (Hamilton *et al.*, 2006; Iyer *et al.*, 1996b). As expected, treatment with CSiO<sub>2</sub> caused significant loss in cell viability while treatment of TiO<sub>2</sub> and ASiO<sub>2</sub> was found to be relatively non-toxic to AM (Figure 9).





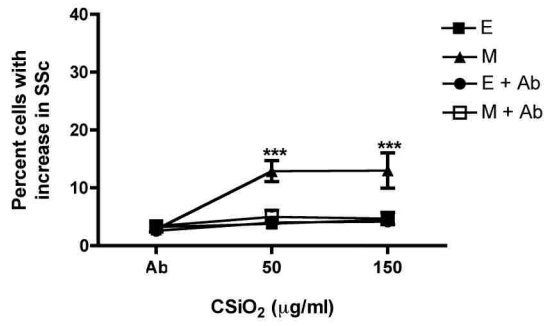
**Figure 9: Effect of inorganic particle treatment on alveolar macrophage viability.**

CSiO<sub>2</sub> induced dose dependent loss in cell viability as compared to untreated AM, while ASiO<sub>2</sub> and TiO<sub>2</sub> treatments did not have effect at any concentration used. Mean ± SE, percent cells excluding trypan blue dye. \*\*\*, indicates p<0.001 compared with untreated AM by Bonferroni's post hoc test. Sample size n=3.

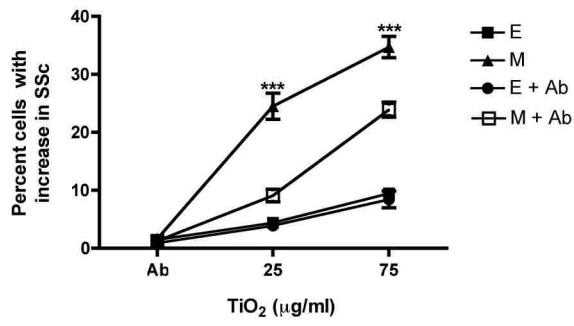
### **Crystalline and Amorphous Inorganic Particles Bind to MARCO *in vitro*.**

The scavenger receptor MARCO is an important player in binding of both toxic CSiO<sub>2</sub> and non-toxic TiO<sub>2</sub> (Hamilton *et al.*, 2006; Palecanda *et al.*, 1999a). Therefore, to begin understanding the role of MARCO in the contrasting effects of these particles, their binding patterns to MARCO were analyzed (Figure 10). Initial studies were performed using CHO cells transiently transfected with full length human MARCO (M) or empty vector, pretreated with or without MARCO antibody (PLK-1) or isotype control IgG3, followed by treatment with different concentrations of the three particles. Binding experiments were performed using a flow cytometric assay (Palecanda and Kobzik, 2000), wherein an increase in mean side scatter intensity was used as a marker for binding of CSiO<sub>2</sub> and TiO<sub>2</sub>. For the DAPI conjugated ASiO<sub>2</sub> particles, increase in percentages of DAPI positive cells was used as a measure of binding. The results showed that all three inorganic particles bound to MARCO (Figure 10). Further to define the specificity of particle bindings to MARCO, binding studies were conducted following pretreatment with MARCO specific blocking antibody (Figure 10). In each case, the MARCO antibody efficiently blocked binding for all three inorganic particles while the isotype control had no effect (Figure 10; data not shown), suggesting that the SRCR domain of MARCO contained the particle binding sites.

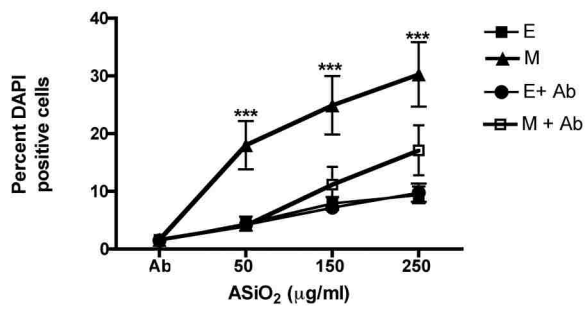
A



B



C



**Figure 10: Binding of CSiO<sub>2</sub>, ASiO<sub>2</sub> and TiO<sub>2</sub> to MARCO.**

All three inorganic particles (A) CSiO<sub>2</sub>, (B) TiO<sub>2</sub> and (C) ASiO<sub>2</sub> significantly bound human MARCO transfected cells and the human MARCO specific antibody (PLK1) inhibited the binding. Results represent mean values  $\pm$  SE of three independent experiments. \*\*\*, indicates  $p < 0.001$  as compared to side scatter (SSc) of corresponding “Ab” treated cells by Bonferroni’s post hoc test.

### **SRCR domain of MARCO is required for binding inorganic particles.**

In order to study the requirement of the SRCR domain of MARCO in binding of particles, CHO cells were transiently transfected with full length or truncated forms of MARCO. The truncated mutants either lacked the entire SRCR domain of MARCO (Mt) or only contained the first 22 amino acid residues of the SRCR domain (M442) that was reported to contain the bacteria binding 'RGR' motif (Brannstrom *et al.*, 2002a). Cell surface expression of the truncated mutants was analyzed by surface biotinylation (Figure 11). CHO cells were transfected with full length and truncated mutants, followed by precipitation of biotinylated proteins on ice and electroblotting as described in the Methods. The nitrocellulose membranes were then probed with an antibody against the intracellular domain of MARCO. The results indicated that the mutants M442 and Mt were expressed on the surface (lanes 2 and 3) at higher levels compared to the full length MARCO (lane 4). The increased expression of the mutants is similar to previously published results (Brannstrom *et al.*, 2002a). As controls, SRA I and mock-transfected cells were probed with the same antibody. As expected, streptavidin-agarose was not found to precipitate any biotinylated MARCO for mock (Lane 5) or SRA I (Lane 1) transfected cells.

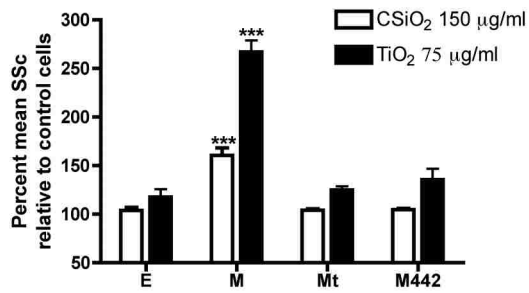
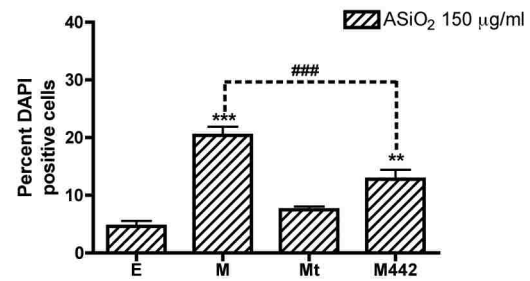
The role of the SRCR domain of MARCO in particle binding was studied using transfected cells incubated with the three inorganic particles measured using flow cytometry as described above. The results showed that cells expressing the mutant lacking the SRCR domain of MARCO (Mt) did not bind any of the particles, indicating that the SRCR domain of MARCO is a common binding domain for all the tested particles. The truncated protein (M442) with the "RGR" motif was not sufficient to bind CSiO<sub>2</sub> and TiO<sub>2</sub> (Figure 12A). However, the ASiO<sub>2</sub> particles bound to M442 expressing cells although the

binding was significantly less than the full length MARCO expressing cells (Figure 12B). Another mutant containing the first 11 amino acids of SRCR domain and lacking the “RGR” motif (M431) did not bind the ASiO<sub>2</sub> particles (data not shown). These results showed that the “RGR” motif (found capable for supporting bacterial binding) in the SRCR domain of MARCO is not sufficient for binding of CSiO<sub>2</sub> and TiO<sub>2</sub> but the motif appears to play a partial role in ASiO<sub>2</sub> binding.



**Figure 11: Expression of MARCO mutants on the cell surface of CHO cells.**

Surface biotinylation was performed on CHO cells expressing empty vector (E), full length MARCO (M), MARCO mutants, (Mt), (M-442) and scavenger receptor A I (SRA I) (control). The nitrocellulose membrane was probed with antibody against the intracellular domain of human MARCO. Mt and M-442 were expressed at a higher level as compared to full length MARCO on the cell surface. The intracellular MARCO antibody recognizes a nonspecific band in the truncated MARCO transfected cell lysates. The Western blot is representative of two experiments.

**A****B**

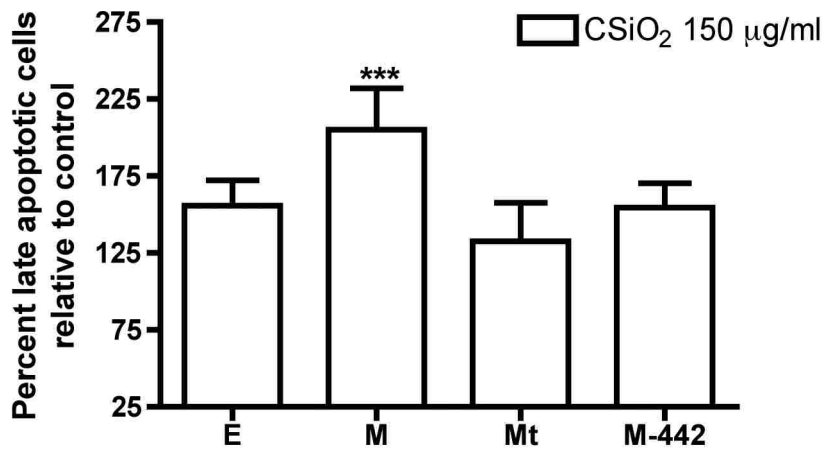
**Figure 12: Binding of inorganic particles by human MARCO requires the SRCR domain.**

(A) The mutant lacking the SRCR domain of MARCO Mt or the M442 mutant did not bind either CSiO<sub>2</sub> or TiO<sub>2</sub>, indicating that SRCR domain of MARCO is the binding domain for these particles and the “RGR” motif does not play a role in binding of these particles. (B) ASiO<sub>2</sub> did not bind Mt expressing cells however ASiO<sub>2</sub> showed partial binding to the M442 expressing cells indicating that compared with CSiO<sub>2</sub> and TiO<sub>2</sub>, the “RGR” motif plays a partial role in ASiO<sub>2</sub> binding. Results represent mean values ± SE. \*\*, indicates p < 0.01; \*\*\*, indicates p < 0.001 compared with “E” control and ###, indicates p < 0.001 compared with corresponding “M-442” cells by Bonferroni’s post-hoc test. Sample size=3-4.



**MARCO is required for CSiO<sub>2</sub>-induced cytotoxicity.**

Crystalline silica (CSiO<sub>2</sub>) has been reported to induce both apoptosis and necrosis in murine and human AM (Iyer *et al.*, 1996b; Iyer and Holian, 1997). In contrast, TiO<sub>2</sub> and ASiO<sub>2</sub> have been reported to be relatively benign (Figure 13). Therefore, in order to confirm the contribution of the SRCR domain in MARCO to cytotoxicity in the transfected CHO cell model, the full length MARCO and MARCO variant transfected cells were treated with CSiO<sub>2</sub> or TiO<sub>2</sub> for 6 h. The cells were stained with 1 μm of YOPRO-1 and PI, and the percentages of viable and apoptotic cells were analyzed by flow cytometry. The results indicated that CSiO<sub>2</sub> induced significant cytotoxicity in full length MARCO transfected cells compared to the mock and Mt transfected cells (Figure 13). In contrast, TiO<sub>2</sub> particles were not cytotoxic to any of the transfectants (data not shown). The results support the role of the SRCR domain of MARCO in binding and CSiO<sub>2</sub>-induced cytotoxicity.



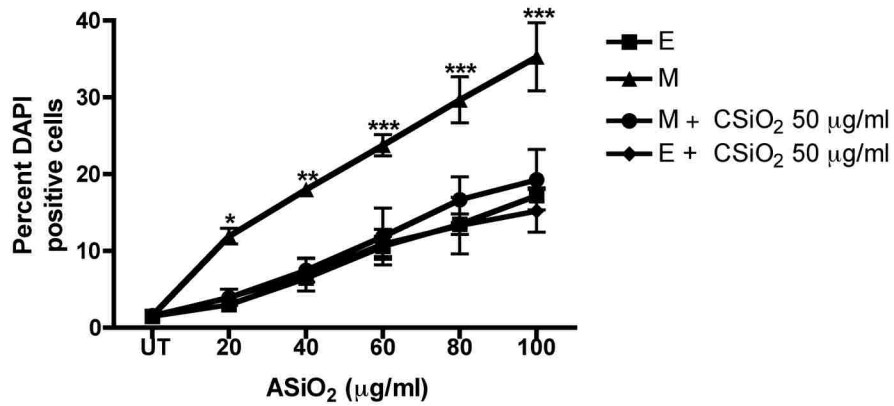
**Figure 13: CSiO<sub>2</sub> induces cytotoxicity in only full length MARCO transfected cells.**

CSiO<sub>2</sub> induced cytotoxicity in only full length MARCO transfected cells. Results represent mean values  $\pm$  SE. \*\*\*, indicates  $p < 0.001$  compared with “Mt” control by Bonferroni’s post-hoc test. Sample size=3-4.

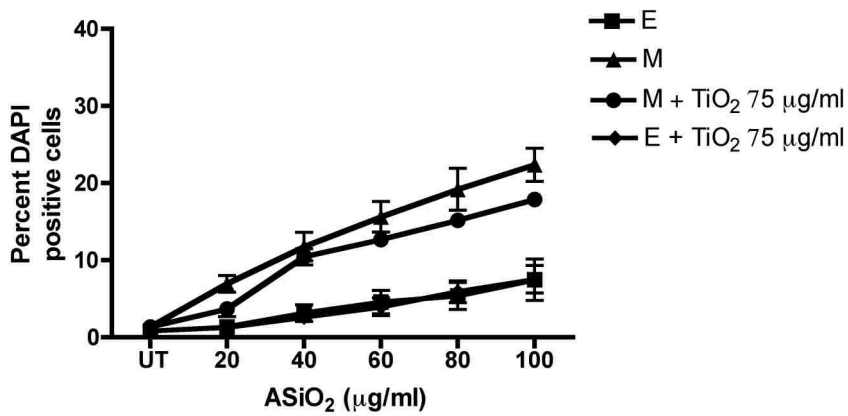
### **Inhibition of ASiO<sub>2</sub> binding by CSiO<sub>2</sub>, but not by TiO<sub>2</sub>.**

Competitive binding studies were conducted to determine if all the tested particles had overlapping binding domains or whether they use different binding motifs in the same SRCR domain. The transfected cells were pretreated with CSiO<sub>2</sub> or TiO<sub>2</sub>, 15 minutes prior to incubation with different concentrations of ASiO<sub>2</sub> particles. CSiO<sub>2</sub> significantly inhibited ASiO<sub>2</sub> binding (Figure 14A). The TiO<sub>2</sub> particles however did not inhibit the binding of ASiO<sub>2</sub> particles to MARCO (Figure 14B). The results strengthened the hypothesis that TiO<sub>2</sub> and CSiO<sub>2</sub> bind to different regions on the SRCR Domain of MARCO. Since all particles increase the side scatter of cells when they bind, the reverse experiment could not be conducted, viz., if ASiO<sub>2</sub> effectively inhibits CSiO<sub>2</sub> binding.

A



B



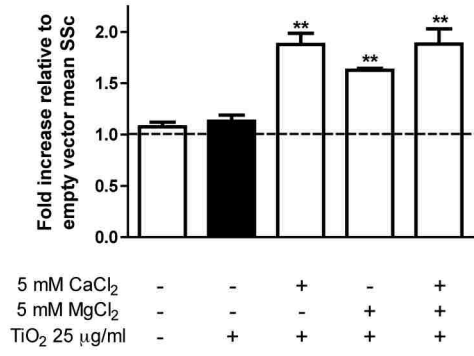
**Figure 14: CSiO<sub>2</sub> and TiO<sub>2</sub> bind to the distinct regions of SRCR domain of MARCO.**

**(A)** CSiO<sub>2</sub> pretreatment completely inhibits ASiO<sub>2</sub> binding. **(B)** In contrast, TiO<sub>2</sub> pretreatment did not inhibit ASiO<sub>2</sub> binding. Results represent mean values  $\pm$  SE. \* p < 0.05; \*\*, indicates p < 0.01 and \*\*\*, indicates p < 0.001 compared with corresponding “particle pretreated M cells” by Bonferroni’s post hoc test. Sample size=3.

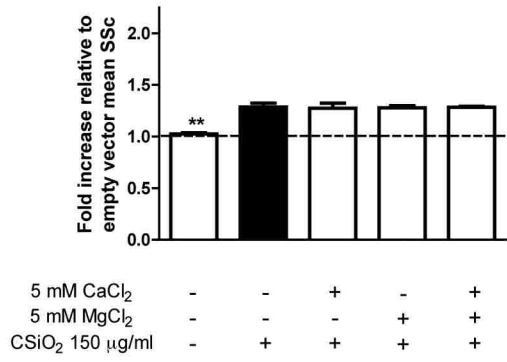
### **Role of divalent cations on particulate binding.**

The crystalline structure of SRCR domain of murine MARCO was recently found to contain  $\text{Mg}^{+2}$  in its acidic amino acid cluster (Ojala *et al.*, 2007). Also, the presence of divalent cations was found to be necessary for binding of some MARCO ligands (Ojala *et al.*, 2007). To date, no studies have been conducted examining the role of divalent cations in particle binding to MARCO. Therefore, the binding of the three particles to MARCO-transfected CHO cells was measured in the presence or absence of exogenous cations (5 mM of  $\text{CaCl}_2$ ,  $\text{MgCl}_2$  or both). The binding of crystalline and amorphous forms of silica was not affected by the presence or absence of divalent cations (Figure 15B-C). In contrast, no significant  $\text{TiO}_2$  binding was observed in the absence of the divalent cations or in the presence of EDTA (added to ensure complete divalent cation removal, see Methods) (Figure 15A). The addition of  $\text{CaCl}_2$  and/or  $\text{MgCl}_2$  allowed the binding of  $\text{TiO}_2$  to MARCO (Figure 15A). These findings indicate that the  $\text{TiO}_2$  interactions with MARCO specifically require divalent cations, while  $\text{CSiO}_2$  and  $\text{ASiO}_2$  do not.

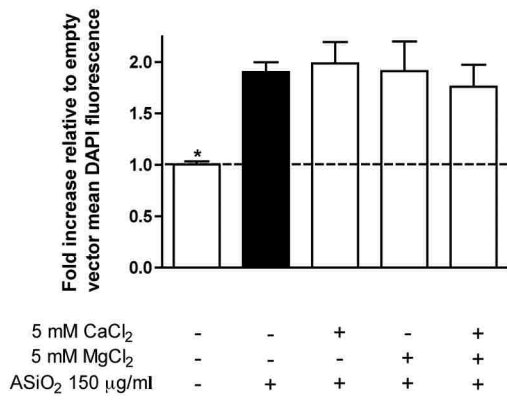
A



B



C



**Figure 15: Effect of divalent cations  $\text{Ca}^{+2}$  and  $\text{Mg}^{+2}$  on  $\text{CSiO}_2$ ,  $\text{ASiO}_2$  and  $\text{TiO}_2$  binding by MARCO.**

(A)  $\text{TiO}_2$  did not bind MARCO transfected CHO cells in the absence of divalent cations, presence of divalent cations in media restored the  $\text{TiO}_2$  binding. **(B and C)** In contrast,  $\text{CSiO}_2$  and  $\text{ASiO}_2$  binding was not dependent on presence of divalent cations. The values are represented as fold increase relative to empty vector mean SSc. Results represent mean values  $\pm$  SE. \*, indicates  $p < 0.05$  and \*\*, indicates  $p < 0.01$  compared with corresponding “mean SSc of particle treated M cells without divalent cations” by Bonferroni’s post-hoc test. Sample size=3.

### **Physical properties of inorganic particles**

In order to better assess the role of physical properties of the particles on their binding, the influence of properties such as particle size, shape and zeta potential (measure of the surface charge) on interaction with MARCO was examined (Table 1). SEM and light scattering analysis showed that the overall diameter of all the inorganic particles ranged from 200 nm to 2.5  $\mu\text{m}$ . The  $\text{TiO}_2$  particles were the smallest and had a uniform diameter of 100-300 nm. The  $\text{ASiO}_2$  particles were 1  $\mu\text{m}$  in size, according to the specifications of vendor (Postnova Analytics). The  $\text{CSiO}_2$  was found to be more heterogeneous in composition with both relatively large and small particles. The large particles were about 1-2.5  $\mu\text{m}$  in size, while the smaller particles ranged from 200 nm – 1.0  $\mu\text{m}$ . The suspending medium (media,  $\text{H}_2\text{O}$ , PAB) had no significant effect on the particle size (Table 1).

Analysis of the SEM data also revealed the shape of the particles. The  $\text{TiO}_2$  particles were found to be spherical, while  $\text{CSiO}_2$  particles were found to be irregular in shape (Table 1). The  $\text{ASiO}_2$  particles, according to the vendor specification (and visual microscopic examination) were spherical in shape. The results obtained from the zeta potential measurement of the particles (Table 1) showed that the  $\text{TiO}_2$  particles were most negative with zeta potential (-) 47.9 mV whereas  $\text{CSiO}_2$  and  $\text{ASiO}_2$  had a similar zeta potential, (-) 16.2 mV and (-) 17.8 mV, respectively.



**TABLE 1** Physical properties of inorganic particles used in the study.

	<b>Particle Size</b>	<b>Shape</b>	<b>Zeta Potential</b>
<b>ASiO<sub>2</sub></b>	<b>1.0 μm</b> <b>SD &lt;0.25</b>	<b>spherical</b>	<b>-17.8</b>
<b>CSiO<sub>2</sub></b>	<b>1-2.5 μm</b> <b>some &lt; 1.0 μm</b>	<b>irregular</b>	<b>-16.2</b>
<b>TiO<sub>2</sub></b>	<b>100-200 nm</b>	<b>spherical</b>	<b>- 47.9</b>

### 3.4. DISCUSSION

Recent studies provided evidence that in AM from C57Bl/6 mice, MARCO plays a predominant role in binding of toxic CSiO<sub>2</sub> particles (Hamilton *et al.*, 2006). The ability of MARCO to bind inert non-toxic TiO<sub>2</sub> particles was first reported by Kobzik and co-workers (Palecanda *et al.*, 1999a). These observations raise an important question as to why, despite binding to a common receptor MARCO, certain inorganic particles such as CSiO<sub>2</sub> are toxic to the AM while TiO<sub>2</sub> particles are not (Figure 9). We hypothesized that the differences in the apoptotic outcome in response to these inorganic particles may, at least in part, be related to differences in binding of these particles to MARCO. To understand the differences in binding of environmental particles to MARCO, the purpose of this study was to define the particle-binding domain of MARCO and map some of the determinants for individual particle binding to MARCO.

To explore the possibility that crystalline and amorphous forms of silica, as well as TiO<sub>2</sub>, bind to distinct motifs in the receptor MARCO, a transfected cell line model was developed. For these studies, CHO cells expressing full length MARCO or various MARCO mutants were used. The cell surface expression of the full length MARCO and mutants was confirmed by cell surface biotinylation (Figure 11). The results of the binding studies conducted with full length MARCO transfected cells showed that all three particles bound to MARCO (Figure 10). The fact that the MARCO specific antibody, which binds to an epitope in the SRCR domain, significantly inhibited the binding of all three particles to MARCO suggested that the SRCR domain was the particle-binding domain of MARCO. This finding was confirmed by the observation that the MARCO mutant without the SRCR domain failed to bind any of the particles (Figure 12A-B). Consequently, the data established that all these particles require the SRCR

domain for binding. Cytotoxicity assays with transfected CHO cells further showed that the CSiO<sub>2</sub> binding to the SRCR domain of MARCO was required for its cytotoxicity (Figure 13), whereas treatment with ASiO<sub>2</sub> and TiO<sub>2</sub> did not induce cytotoxicity in the full length MARCO transfected cells (data not shown) despite efficient binding (Figure 10). The results support the hypothesis that the SRCR domain is the binding domain for environmental particles.

The next question was whether the “RGR” motif within the SRCR domain would be sufficient for particle binding. The M442 mutant containing the “RGR” motif showed significant ability (distinctly reduced compared to full length MARCO) to bind only the ASiO<sub>2</sub> particles (Figure 12B). The CSiO<sub>2</sub> and TiO<sub>2</sub> particles did not bind to the M442 mutant expressing cells. The “RGR” motif within the SRCR domain has been previously shown to be sufficient for bacterial binding (Brannstrom *et al.*, 2002a). Importantly, this finding suggests that ASiO<sub>2</sub> is unique with respect to the “RGR” motif in contrast to CSiO<sub>2</sub> and TiO<sub>2</sub> and hence binds distinctly to MARCO.

Competitive binding studies were conducted to further investigate how each particle binds to the SRCR domain of MARCO. For these studies cells were pretreated with CSiO<sub>2</sub> or TiO<sub>2</sub> particles prior to incubation with ASiO<sub>2</sub> particles. Considering relatively large sizes of the particles the complete inhibition of ASiO<sub>2</sub> binding by CSiO<sub>2</sub> was not unexpected (Figure 14A). Furthermore, this observation does not negate the proposed role of the “RGR” motif as being sufficient for ASiO<sub>2</sub> binding. It should be kept in mind while interpreting these results that all of these particles are very large with respect to MARCO. Therefore, the observation that TiO<sub>2</sub> did not completely block ASiO<sub>2</sub> binding is more difficult to explain (Figure 6B). Nevertheless, it indicates a divergence in

the requirements between both the silica particles and TiO<sub>2</sub> in binding to MARCO. This is not a classic case of ligand receptor binding, but may require multiple MARCO receptors interacting with these rather large ligands.

Divalent cation binding properties of certain receptors such as LDL receptors is often exploited in nature to regulate complex biological events such as receptor-ligand interaction, endocytosis and dissociation of the ligand from the receptor (Dirlam-Schatz and Attie, 1998). Recently, the SRCR domain of MARCO was shown to contain an acidic and a distinct basic cluster of amino acids, both the clusters were reported to be important for ligand binding. The crystallized SRCR domain of MARCO contained a divalent cation in the acidic cluster (Ojala *et al.*, 2007). In the current study, divalent cations (Ca<sup>+2</sup>, Mg<sup>+2</sup>) were necessary only for TiO<sub>2</sub> binding (Figure 15A), whereas the other two inorganic particles (CSiO<sub>2</sub> or ASiO<sub>2</sub>) did not depend on the presence of divalent cations (Figure 15B-D). Calcium binding to the cysteine-rich domain of a particular protein has been shown to stabilize the protein conformation (Handford *et al.*, 1990; Knott *et al.*, 1996; Thielens *et al.*, 1988). Bound calcium might cause a conformational change in the SRCR domain and expose certain amino acid residues leading to more efficient binding. The data suggests that either the TiO<sub>2</sub> binds to the acidic cluster (containing the divalent cations) of the SRCR domain or the divalent cation binding to the SRCR domain leads to a distinct conformational changes in the binding domain facilitating TiO<sub>2</sub> binding. It should be noted that changes in dispersion medium such as divalent cations will affect the zeta potential of all three particles. However, addition of divalent cations would affect all particles in a relatively similar manner. Therefore, it is most likely that the divalent cations in this study act on the SRCR domain

of MARCO. Taken together, the results (Figure 12, 14 and 15) emphasize that the three different particles in the study show significant differences in binding to MARCO.

The differences in the pathological outcomes after exposure to each particle are speculated to be related to the differences in the physical properties of the particles. (Johnston *et al.*, 2000; Thakur *et al.*, 2008). Therefore, the particles were characterized and analyzed for their size, shape and surface charge (Table 1). The analysis suggested that there were no major differences in size of the particles. The overall surface charge or the zeta potential of the silica particles was essentially identical (-) 16.2 to (-) 17.8. The TiO<sub>2</sub> particles were the most negative particles with zeta potential (-) 47.9. While the net surface charge of the particles could be measured by zeta potential measurements, the surface charge distribution (order) could not be determined. While it appears that TiO<sub>2</sub> differs from the two silica particles in how it binds to MARCO and therefore could explain the difference in toxicity between TiO<sub>2</sub> and CSiO<sub>2</sub>, the difference between ASiO<sub>2</sub> and CSiO<sub>2</sub> appears to be subtle. They are similar in relative size and surface charge, but differ in shape and crystal structure (Table 1). It is possible that one or both the properties contribute to the difference in toxicity between the two silica particles. The only difference in binding appeared to be the sufficiency of the “RGR” motif for ASiO<sub>2</sub> binding. The assumption made in these studies is that the two silica particles cause difference in conformational change in MARCO such that CSiO<sub>2</sub> causes cytotoxicity while ASiO<sub>2</sub> does not. As stated above, the difference in shape and crystal structure could affect silica binding to MARCO causing differences in conformation and downstream signaling. Without more data, the importance of shape or crystal structure is speculative. As yet the signaling pathways initiated by receptor MARCO are not completely

elucidated, which makes the aforementioned theory difficult to test experimentally.

We propose that factors such as presence of divalent cations, shape of the particle and crystal structure (the distribution or the order of surface charge) on the particle may play an important role in determining toxic or non-toxic binding of particles. Studies conducted with more diverse sizes and surface charge, of one particle could further strengthen these conclusions. Nevertheless, the results obtained in the current study provide strong support to the notion that environmental particles bind to distinct motifs in the SRCR domain of MARCO, which is influenced by individual physical properties. The implications of these observations are that each particle-MARCO interaction may trigger unique conformational changes in the receptor, which might influence the recruitment of different intracellular proteins leading to diverse biological responses.

### 3.5. REFERENCES

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## CHAPTER THREE

### CRITICAL ROLE OF MARCO IN CRYSTALLINE SILICA-INDUCED PULMONARY INFLAMMATION

#### ABSTRACT

Exposure to crystalline silica (CSiO<sub>2</sub>) is a serious occupational hazard and has been reported to lead to inflammation that progresses to silicosis. Previous studies have established the scavenger receptor MARCO as an important receptor for binding and uptake of CSiO<sub>2</sub> particles. Although MARCO is responsible for binding CSiO<sub>2</sub>, the role of MARCO in inflammatory response following CSiO<sub>2</sub> exposure has not been investigated. We hypothesize that absence of MARCO will cause diminished clearance of CSiO<sub>2</sub> from the lung leading to enhanced pulmonary inflammation. Consistent with the *in vitro* studies, alveolar macrophages (AM) from MARCO<sup>-/-</sup> mice show decreased particle uptake *in vivo*, as compared to WT mice, indicating abnormalities in clearance mechanisms. Furthermore, MARCO<sup>-/-</sup> mice showed enhanced acute inflammation and injury, following CSiO<sub>2</sub> treatment marked by significant increases in cytokines and inflammatory cells. Similarly, histological analysis of lungs from MARCO<sup>-/-</sup> mice, three months post- CSiO<sub>2</sub> exposure showed increased inflammatory cells in the tissue sections as compared to the C57Bl/6 WT mice. As hypothesized, the results demonstrate that MARCO is important for clearance of fibrogenic CSiO<sub>2</sub> particles and absence of MARCO causes exacerbations in acute and chronic inflammatory responses. Furthermore, results from *in vitro* studies suggest that coating CSiO<sub>2</sub> particles with His tagged recombinant MARCO (rMARCO) facilitates anti His-tag antibody mediated

particle uptake by Fc receptors on AM. Additional, *in vivo* studies analyzing the effect of rMARCO on enhanced inflammatory response in CSiO<sub>2</sub>-exposed MARCO<sup>-/-</sup> mice will evaluate the therapeutic potential of rMARCO.

#### **4.1. INTRODUCTION**

Exposure to respirable particles such as crystalline silica (CSiO<sub>2</sub>), is associated with an increase in pulmonary inflammation, which plays a vital role in pathologies such as bronchitis and pulmonary fibrosis known as silicosis (Park *et al.*, 2002). Exposure to CSiO<sub>2</sub> also results in the development of autoimmune diseases such as scleroderma and systemic lupus erythematosus (Brown *et al.*, 2005; Parks *et al.*, 2002). Most investigators agree that alveolar macrophages (AM) play a central role in CSiO<sub>2</sub>-induced pathologies (Hamilton *et al.*, 2008; Lehnert *et al.*, 1989). AM are purported to be important in the innate defense of the lung against inhaled particles and play a critical role in the recognition, uptake and clearance of CSiO<sub>2</sub> particles via the mucociliary escalator and/or lymphatic systems (Bowden, 1987; Brody *et al.*, 1982).

Balance between clearance and retention of CSiO<sub>2</sub> in the lung plays an important role in regulating the inflammatory response and development of pulmonary fibrosis. Also, facilitating the clearance of CSiO<sub>2</sub> from the alveolar and interstitial compartments decreases the fibrotic response in the lung (Adamson *et al.*, 1992, 1994). Unsuccessful clearance of CSiO<sub>2</sub> results in persistent inflammation, due to prolonged interaction of CSiO<sub>2</sub> with immune cell populations such as neutrophils, AM, dendritic and epithelial cells, leading to epithelial cell injury and translocation of uncleared particles to the interstitial space (Warheit *et al.*, 1997). The CSiO<sub>2</sub>- interstitial macrophage interaction initiates a cascade of inflammatory signals, which are major contributors to progressive

fibrotic development in the lung (Adamson *et al.*, 1991). The above observations highlight the importance of initial recognition and rapid clearance of CSiO<sub>2</sub> from the lung, to minimize the inflammatory response by AM.

Alveolar macrophages recognize and bind CSiO<sub>2</sub> particles by class A scavenger receptors expressed on their surface (Hamilton *et al.*, 2006; Thakur *et al.*, 2008). The Class A scavenger receptors (SR) are pattern recognition receptors that bind a wide variety of ligands such as acetylated low density lipoprotein (AcLDL), bacteria, inhaled particles and are known to play a role in innate immune responses (Murphy *et al.*, 2005; Thakur *et al.*, 2008). Scavenger receptors are mainly expressed on macrophages, dendritic and epithelial cells (Sarrias *et al.*, 2004). The Class A family of scavenger receptors is composed of five family members; SRA (SRA -I, -II and -III), MARCO, CSR1 (cellular stress response 1), SRCL (Scavenger receptor with C-type lectin) and SCARA 5 (class A scavenger receptor 5) (Thakur *et al.*, 2008). Of the five identified Class A SR, the macrophage receptor with collagenous structure (MARCO) has been previously shown to be important for binding of unopsonized particles such as CSiO<sub>2</sub> and titanium dioxide (TiO<sub>2</sub>) (Arredouani *et al.*, 2004; Hamilton *et al.*, 2006). In particular, the C-terminal 100 amino acid long cysteine rich (SRCR) domain of MARCO has been reported as the binding region for CSiO<sub>2</sub> particles (Chapter 2).

The SRCR domain is an ancient and highly conserved motif and found as a single or tandem repeats in several soluble or membrane bound proteins often associated with the innate immune system (Sarrias *et al.*, 2004). Recently, a soluble recombinant MARCO protein composed of the C-terminal extracellular domain of MARCO, but lacking the N-terminal cytoplasmic and transmembrane domains (rMARCO) was shown

to efficiently bind ligands such as bacteria and AcLDL in a cell free system (Chen *et al.*, 2006; Sankala *et al.*, 2002). Previous work has identified a specific role for MARCO in regulation of titanium dioxide (TiO<sub>2</sub>) induced acute inflammatory response (Arredouani *et al.*, 2004). While MARCO has been shown to be important for lung defense against the non-fibrogenic TiO<sub>2</sub> particles, its physiological role in regulating the inflammatory response against the fibrogenic CSiO<sub>2</sub> particles has not been reported.

In this study, we examined the role of MARCO in clearing CSiO<sub>2</sub> particles from the lung and investigated whether MARCO-mediated clearance plays a role in reducing the inflammatory response. We hypothesize that absence of MARCO will diminish the clearance of CSiO<sub>2</sub> from the lung leading to increased lung injury and inflammatory response. We also explored the possibility of facilitating CSiO<sub>2</sub> clearance through an alternative mechanism, which may have therapeutic implications for subjects that have diminished clearance of inhaled particles from the lung. Taken together, the role of MARCO in pulmonary response following CSiO<sub>2</sub> exposure was characterized *in vivo* using C57Bl/6 wild type and MARCO<sup>-/-</sup> mice.

## **4.2. MATERIALS AND METHODS**

### **Mice**

Breeding pairs of C57Bl/6 and Balb/c mice were originally purchased from The Jackson Laboratory (Bar harbor, ME, USA); while breeding pairs of MARCO<sup>-/-</sup> mice on C57Bl/6 background were kindly provided by Dr. Lester Kobzik (Harvard School of Public Health, Boston, MA). Age-matched (6-8 weeks), males and females were used for all the studies. Genotyping was carried out as described previously (Dahl *et al.*, 2007).

All mice were maintained in the University of Montana specific pathogen-free (SPF) laboratory animal facility. The mice were maintained on an ovalbumin-free diet and given deionized water *ad libitum*. The University of Montana Institutional Animal Care and Use Committee (IACUC) approved all animal procedures.

## **Particles**

Crystalline silica (CSiO<sub>2</sub>), Min-U-Sil-5, obtained from Pennsylvania sand glass corporation (Pittsburg, PA, USA), was acid washed in 1 M HCl at 100°C, to remove metals and any endotoxin contamination. The CSiO<sub>2</sub> particles were then washed three times with sterile water and dried in oven at 200°C to remove all water. Titanium dioxide (TiO<sub>2</sub>) particles were purchased from Fischer scientific (Pittsburg, PA, USA). DAPI conjugated amorphous silica (ASiO<sub>2</sub>) particles (1 µm in diameter) were purchased from Postnova Analytics, Inc. (Salt Lake City, UT, USA). The ASiO<sub>2</sub> particles were washed three times with sterile phosphate buffered solution (PBS) to remove the shipping medium. The stock suspensions in PAB buffer (PBS buffer containing 0.1% sodium azide and 2 % fetal calf serum) were dispersed by sonic disruption for 1 min before each experiment.

## **Isolation of the interstitial leukocytes**

C57Bl/6 (wild type) or Balb/c mice (8-12 weeks old) were anaesthetized with 0.1 mg/kg of ketamine hydrochloride (Fort Dodge Animal Health, Fort Dogdge, IA, USA) and intranasally instilled with 25 µl of sterile PBS or 1 mg of CSiO<sub>2</sub> or TiO<sub>2</sub> suspended in 25 µl of sterile PBS. Following 3, 7 and 14 days post-instillations, the mice were euthanized with a lethal dose of pentobarbital sodium. Lungs were removed, briefly suspended in sterile PBS on ice and minced with sterile razor blades. The minced lungs

were dispersed in (~5 ml/lung) collagenase buffer containing of 1 mg/ml collagenase 1A (Sigma Chemical Co., St. Louis, MO, USA) in media, (RPMI, Mediatech Herndon, VA, USA) supplemented with 10 % FBS and 1% Pen/Strep, (Gibco, Grand Island, NY, USA) for 1.5 - 2 h. The tubes were then incubated in a 37°C water bath and shaken intermittently. Tubes containing the digested lungs were removed and filtered through sterile cell strainer (BD Biosciences). Post collagenase treatment, multiple cell populations were fractionated using Percoll (GE Biosciences, Piscataway, NJ, USA) gradient centrifugation. The layer between heavy and light percoll solutions was carefully collected with sterile Pasteur pipette avoiding the top (epithelial cells and fibroblasts) and bottom (red blood cells) layers. The collected cell suspension was washed with sterile PBS and resuspended in 1 ml PBS and enumerated using a Z1 Coulter particle counter (Beckman Coulter, Fullerton, CA).

### **Cytokine ELISA**

At 24 h post-particle instillations, wild type (WT) and MARCO<sup>-/-</sup> mice were euthanized with Euthazol and a whole lung lavage was performed by cannulating the trachea and infusing the lungs with sterile PBS four times. The lavage fluid fraction from the first pull was centrifuged (1500 rpm for 5 min at 4°C) and the cell free supernatant was used for biochemical measurements. The cell pellet was combined with the remaining three lavage fractions and total cell numbers were determined. IL-1 $\beta$ , TNF- $\alpha$  and IL-6 concentrations were measured using murine ELISA kits according to manufacturers protocol (R&D Systems Minneapolis, MN, USA).

### **Pulmonary vascular permeability**



Lavage fluid protein from WT and MARCO<sup>-/-</sup> PBS or CSiO<sub>2</sub> exposed mice were analyzed for total protein using bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) according to manufacturers instructions and analyzed with the Spectra Max 340 plate reader (GE Healthcare, United Kingdom). Data are expressed as µg total protein per milliliter of lavage fluid.

### **Flow cytometry**

One x 10<sup>5</sup> lavaged cells or interstitial leukocytes were blocked with purified rat anti-mouse CD16/CD32 BD Pharmingen (San Jose, CA, USA) diluted 1:100 in PAB (PBS buffer with 0.01% sodium azide and 1% fetal calf serum). The lavage cells were stained for cell surface markers with one microgram of monoclonal antibodies specific to CD11c (APC), CD11b (PerCP Cy5.5), Gr-1 (APC Cy7) (BD Biosciences, San Jose, CA, USA) MHC II (PE, eBiosciences, San Diego, CA, USA), F480 (Pacific blue, Catlag Laboratories, Burlingame, CA) for 30 minutes on ice. Interstitial leukocytes were additionally stained with 1 µg of MARCO FITC (Serotec, Raleigh, NC, USA). Cells were washed, resuspended in PAB and analyzed immediately. Acquisition and analysis was performed on a FACS Aria flow cytometer using FACS Diva software (version 4.1.2, BD Biosciences). Compensation of the spectral overlap for each fluorochrome was calculated using anti-rat/hamster Ig compensation beads (BD Biosciences).

### **Determination of lung wet weight and histopathological analysis of lung tissue**

C57Bl/6 (WT) and MARCO<sup>-/-</sup> mice were intranasally instilled with 25 µl PBS or 1 mg CSiO<sub>2</sub>, once a week for 4 weeks, and allowed to recover for three months. Lung weights were determined by weighing unlavaged lungs. The right lobe of the lung was

inflated with 1 ml of 4% paraformaldehyde in PBS and post-fixed for 24 hours at 4°C. Routine histological procedures were used to paraffin embed the lobe. As described previously, five micron sections were cut, mounted on superfrost slides (VWR), and stained with Gomori's trichrome (Beamer and Holian, 2005a). Five mice per group were examined microscopically and representative images captured with a Nikon E-800 microscope and Nikon DXM 1200 digital color camera using 4X and 40X objectives.

### **Effect of antibody mediated uptake of CSiO<sub>2</sub> particles by AM**

The CSiO<sub>2</sub> particles (100 µg/ml) were coated with or without 3 µg of recombinant soluble MARCO with a His tag (rMARCO) protein (R&D Systems) at 37°C for 30 min. These coated silica particles were then treated with or without 3 µg of Anti-His<sub>6</sub> Antibody (Roche-Applied Sciences, Palo Alto, CA) for 30 min at 37°C. Five hundred thousand cells from WT mice were added into the respective eppendorf tubes for one hour on the rotator at 37°C. To determine the role of Fc receptor in CSiO<sub>2</sub> uptake, the cells were incubated with (1:50) Fcblock on ice for 20 min followed by incubation with coated and uncoated CSiO<sub>2</sub> particles. The CSiO<sub>2</sub> uptake was measured by analyzing the increase in mean side scatter or granularity of the AM as described previously (Hamilton *et al.*, 2006) on a FACS Aria flow cytometer using FACS Diva software (version 4.1.2, BD Biosciences).

### **Statistical Analysis**

Differences between CSiO<sub>2</sub> treated and control (PBS or TiO<sub>2</sub>) treated WT and MARCO<sup>-/-</sup> mice were assessed using using the Prism Software, version 4 (GraphPad Prism, San Diego, CA). The significance of differences between treatment groups and

controls was determined using t-test or two-way analysis of variance in conjunction with Bonferroni's post hoc analysis depending on the experiment. Data are represented as mean  $\pm$  SE. A value of  $p < 0.05$  was considered significant.

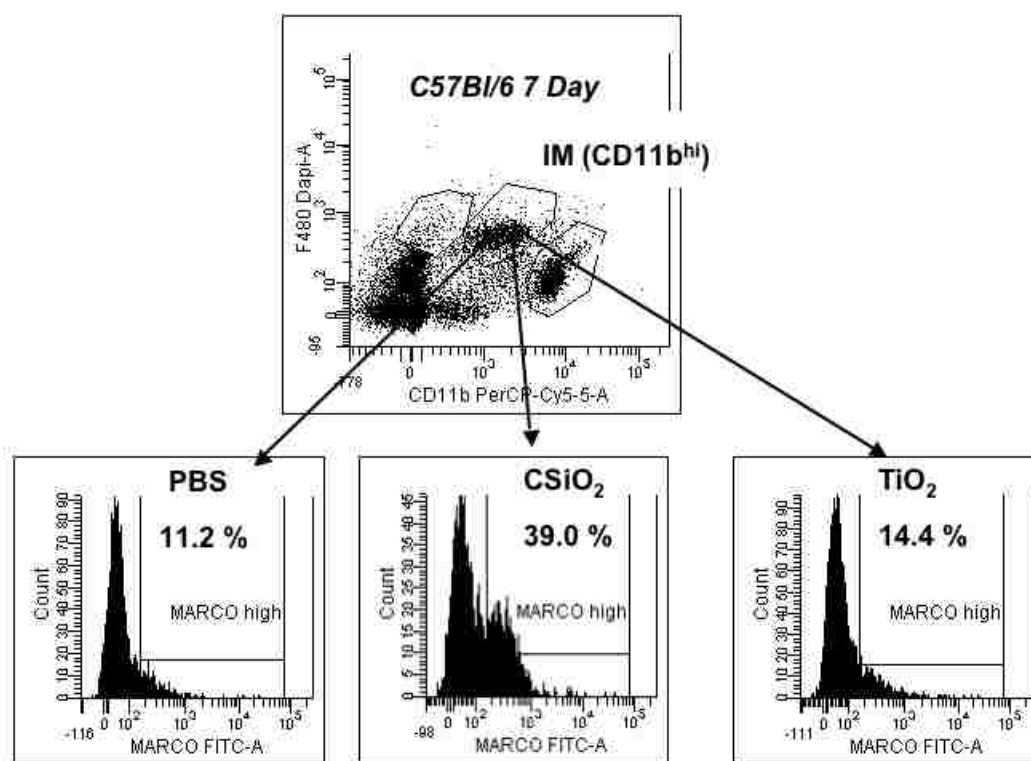
### 4.3. RESULTS

#### MARCO positive interstitial leukocytes

MARCO has been reported to play an important role in inflammation induced by ligands such as bacteria and LPS. Also, MARCO expression has been reported to be increased on several macrophage populations following exposure to these ligands (van der Laan *et al.*, 1999). Therefore, to determine whether intranasal instillations of crystalline silica (CSiO<sub>2</sub>) altered MARCO expression on the lung interstitial macrophages (IM), C57Bl/6 and Balb/c mice were treated with 25  $\mu$ l of PBS, 1 mg of CSiO<sub>2</sub> or TiO<sub>2</sub> (non-fibrogenic particle control) in 25  $\mu$ l of PBS. Three, seven and fourteen days following treatments, individual mouse lungs were digested with collagenase and interstitial leukocytes were isolated, stained with prescribed cells surface markers and analyzed by flow cytometry. Staining for F480 and CD11b cell surface markers was used to identify the previously described subsets of interstitial leukocytes and MARCO expression was assessed in these populations (Figure 16) (Migliaccio *et al.*, 2005). Flow cytometry analysis demonstrated significant increases in percentages of MARCO<sup>+</sup> C57Bl/6 IM (F480<sup>+</sup>/CD11b<sup>hi</sup>) at 7 and 14 days following CSiO<sub>2</sub> instillations when compared with PBS and TiO<sub>2</sub> instillations (Figure 16 and 17A). Similar increases in MARCO<sup>+</sup> IM were observed in Balb/c mice following CSiO<sub>2</sub> instillations relative to the controls, PBS and TiO<sub>2</sub> but with important differences (Figure 17B). In contrast to C57Bl/6, the Balb/c mice showed significant increase in MARCO<sup>+</sup> IM as early as three

days post-CSiO<sub>2</sub> exposure (Figure 17 A and B). Also, there was relatively higher increase in percentages of CSiO<sub>2</sub>-induced MARCO<sup>+</sup> IM in Balb/c mice (~350%) as compared with C57Bl/6 mice (~225%) at 7 and 14 days following CSiO<sub>2</sub> instillations (Figure 17B). The varying percentages of MARCO<sup>+</sup> IM in C57Bl/6 and Balb/c mice following CSiO<sub>2</sub> treatment are suggestive of strain specific differences in importance of MARCO.

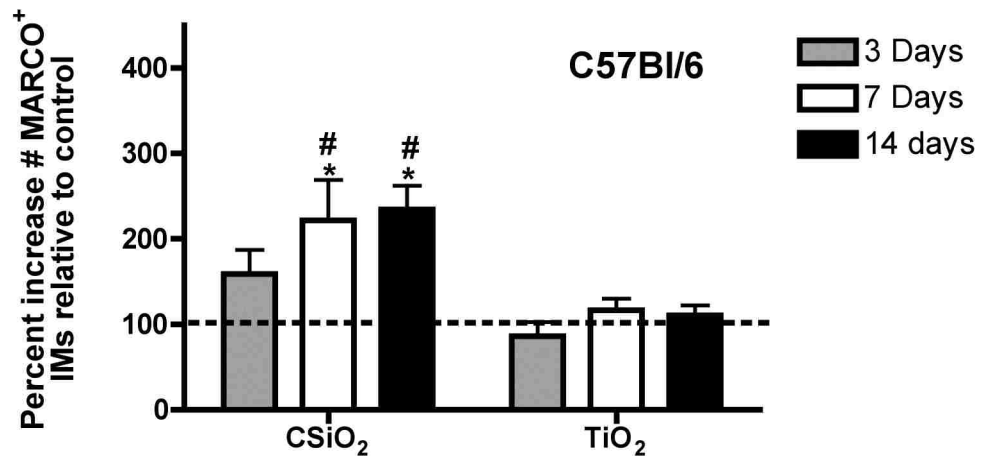
Another intriguing observation is that TiO<sub>2</sub> instillations failed to induce increase in MARCO<sup>+</sup> IM population in both C57Bl/6 and Balb/c mice at all the tested time-points (Figure 17 A and B). This observation suggests differences in signaling mechanisms following CSiO<sub>2</sub> and TiO<sub>2</sub> exposure contributing to varying regulation of MARCO. Altogether, these data show that CSiO<sub>2</sub> exposure upregulates MARCO expression on IM from both C57Bl/6 and Balb/c, while the PBS and TiO<sub>2</sub> exposure does not.



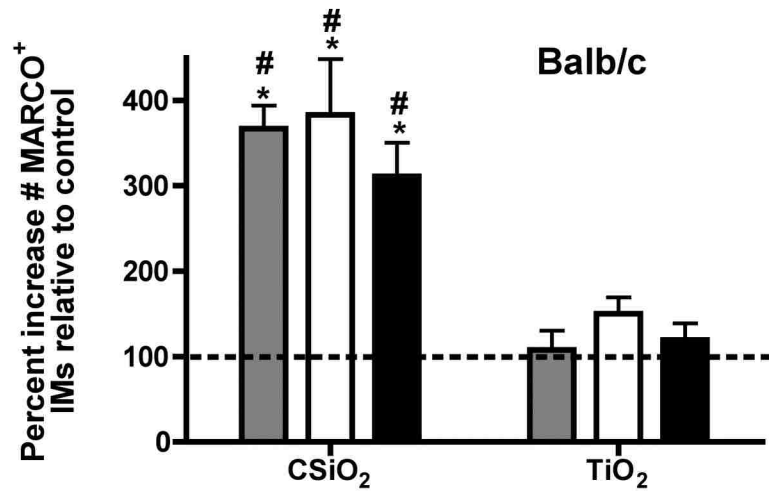
**Figure 16: Representative scatter plot and histograms showing changes in MARCO expression on C57Bl/6 IM.**

C57Bl/6 mice were instilled intranasally with either PBS, CSiO<sub>2</sub> or TiO<sub>2</sub>, at 3, 7 and 14 days, lungs were digested with collagenase and the interstitial leukocytes were isolated by percoll gradient centrifugation. Three distinct subsets were defined by using fluorescent phenotypic markers of which the F480<sup>+</sup>/CD11b<sup>hi</sup> population showed significantly increased MARCO expression following CSiO<sub>2</sub> instillations (second representative histogram) as compared with saline and TiO<sub>2</sub> instillations (first and third representative histogram respectively). Sample size n=4-8.

A



B



**Figure 17: Percent increase in MARCO positive interstitial macrophages following CSiO<sub>2</sub> but not TiO<sub>2</sub> instillation**

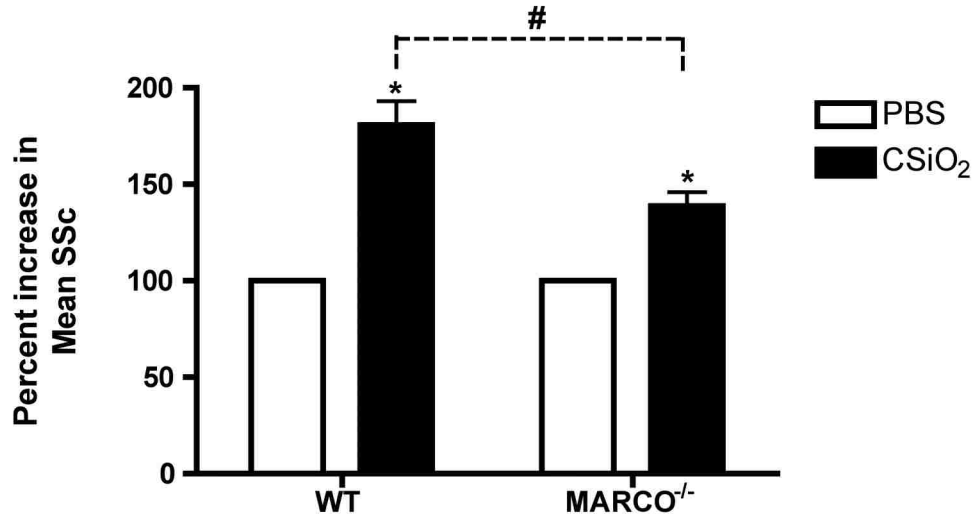
**A.** Relative to PBS instilled control the number of MARCO positive interstitial macrophages (IM) is significantly increased at 7 and 14 days, but not 3 days following CSiO<sub>2</sub> treatment in C57Bl/6 mice. In contrast, TiO<sub>2</sub> exposure had no effect on percent of MARCO positive macrophages at any time point examined (n=4-8). **B.** Similarly, CSiO<sub>2</sub> treatment in Balb/c mice led to increase in percent MARCO positive macrophages at 3, 7 and 14 days whereas TiO<sub>2</sub> had no effect at any time point. The results represent percent increase relative to percentage of MARCO<sup>+</sup> IM from PBS instilled mice and values are reported are mean ± SE for each treatment group (n=4-8). \*, indicates p<0.05 compared to PBS; #, p<0.05 compared to TiO<sub>2</sub> by Bonferroni's post-hoc test.

### **MARCO-mediated uptake and clearance of CSiO<sub>2</sub> from the lung:**

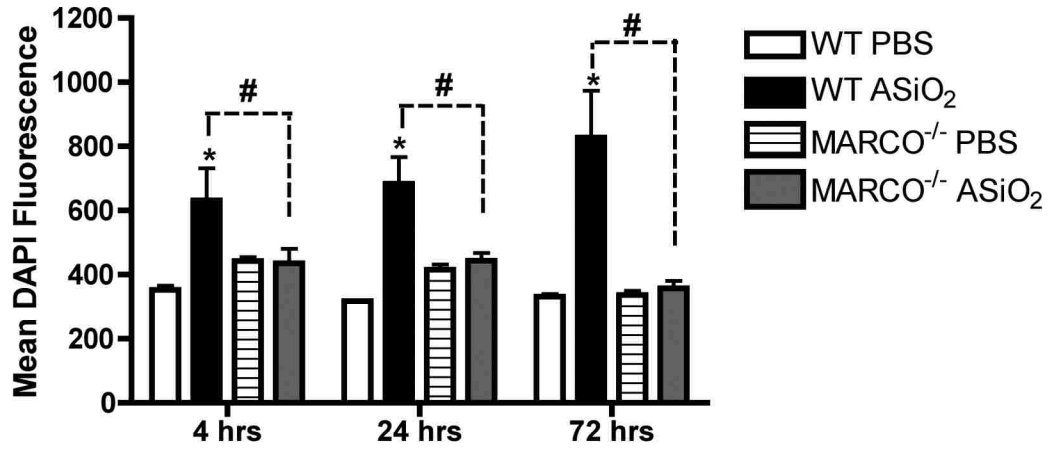
To study the role of MARCO in CSiO<sub>2</sub> recognition and uptake, WT and MARCO<sup>-/-</sup> mice were intranasally exposed to CSiO<sub>2</sub> particles. Individual mouse lungs were lavaged and the cells collected, stained with prescribed cell surface markers and analyzed using flow cytometry. Lavages from naïve C57Bl/6 mice contained approximately 90% of cells, which expressed high levels of CD11c (CD11c<sup>+</sup>). Additional staining of cells for MHC II expression revealed two distinct subsets of CD11c<sup>+</sup> cells. The CD11c<sup>+</sup> cells that expressed low amounts of MHC II were classified as AM, while the CD11c<sup>+</sup> cells with high levels of MHC II expression were classified as DC. Since AM are the cells that are primarily responsible for initial uptake of CSiO<sub>2</sub> particle, changes in the side scatter properties of AM, which are indicative of changes in cellular granularity, were measured by flow cytometry (Hamilton *et al.*, 2006). AM from MARCO<sup>-/-</sup> mice showed attenuated uptake of CSiO<sub>2</sub> particles as compared to WT mice (Figure 18A). Further, DAPI conjugated amorphous silica (ASiO<sub>2</sub>) particles were used to study the role of AM in uptake of silica particles over time (Figure 18B). An increase in mean fluorescence intensity of AM following DAPI ASiO<sub>2</sub> exposure served as a sensitive measure for studying particle uptake as compared to using side scatter changes for CSiO<sub>2</sub> particles. The importance of MARCO in uptake of DAPI conjugated ASiO<sub>2</sub> particles, over time was confirmed at 4, 24 and 72 hours post-instillation (Figure 18B). At all time points investigated, only WT AM bound the DAPI ASiO<sub>2</sub> particles while MARCO<sup>-/-</sup> AM were unable to bind ASiO<sub>2</sub>. These results highlight the role of MARCO in silica uptake and clearance from the lung.



A



B

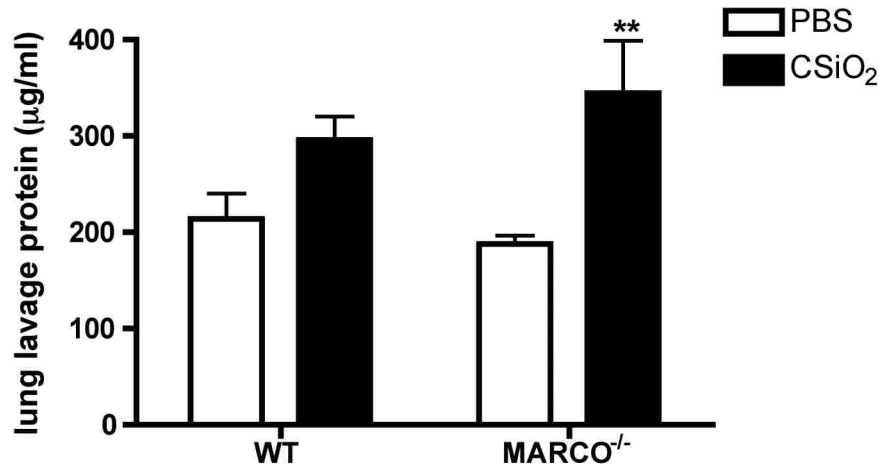


**Figure 18: Silica uptake is attenuated in MARCO<sup>-/-</sup> macrophages**

**(A)** MARCO<sup>-/-</sup> or WT mice were treated intranasally with CSiO<sub>2</sub> (1 mg) or PBS. After 24 h of CSiO<sub>2</sub> treatment, AM from both WT and MARCO<sup>-/-</sup> mice show significant increase in side scatter. However, AM from MARCO<sup>-/-</sup> mice show a significantly attenuated change in side scatter compared to AM from WT mice. **(B)** MARCO<sup>-/-</sup> and WT mice were intranasally instilled with fluorescent ASiO<sub>2</sub> particles. At 4, 24 and 72 h, the AM were analyzed for increase in mean fluorescence intensity (MFI) as a marker of ASiO<sub>2</sub> uptake. Only AM from ASiO<sub>2</sub> treated WT mice showed significant increases in MFI, while AM from ASiO<sub>2</sub> treated MARCO<sup>-/-</sup> mice did not show any change in MFI. Results are reported as mean values ± SE. \*, p<0.05, compared to PBS treated mice,; #, p<0.05, compared to silica treated by Bonferroni's post-hoc test (n=5-6).

### **Assessment of lung injury following CSiO<sub>2</sub>-exposure.**

Diminished uptake and clearance of CSiO<sub>2</sub> particles from the lung by AM leads to increased lung injury and permeability (Driscoll *et al.*, 1991). Lung injury marked by protein leak across alveolar-capillary barrier is often assessed by measuring total protein levels in lavage fluid from mice exposed to environmental toxins (Kenyon *et al.*, 2002). To investigate if decreased CSiO<sub>2</sub> uptake and clearance by AM leads to increased lung injury and permeability, total protein levels in the lavage fluid from MARCO<sup>-/-</sup> and WT mice was measured following 24 h of intranasal exposure to CSiO<sub>2</sub> and PBS (Figure 19). Both WT and MARCO<sup>-/-</sup> mice exposed to CSiO<sub>2</sub> showed an increase in protein content however only MARCO<sup>-/-</sup> mice demonstrated statistically significant changes, indicating enhanced lung injury (Figure 19).

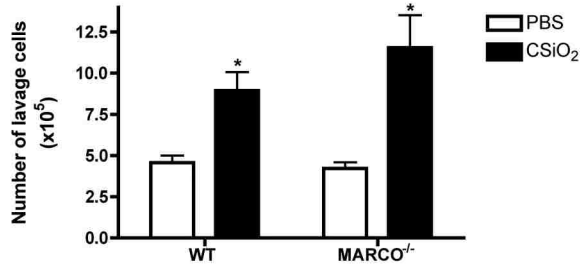
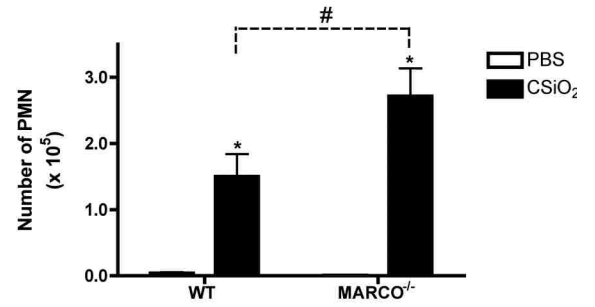
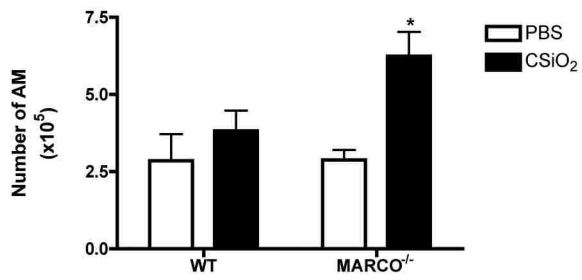
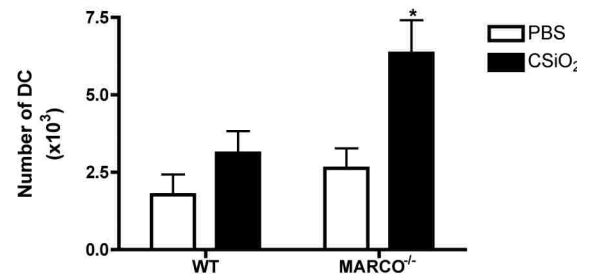


**Figure 19: Enhanced lung injury following CSiO<sub>2</sub> treatment in MARCO<sup>-/-</sup> mice**

Following CSiO<sub>2</sub> exposure lavage fluid from MARCO<sup>-/-</sup> and WT mice was assessed for changes in total protein levels. Both C57Bl/6 WT and MARCO<sup>-/-</sup> mice showed increases in lavage protein levels, however significant changes were observed only in MARCO<sup>-/-</sup> mice. The results are reported as mean ± SE (n=5-6). \*\*p <0.01 compared to PBS treated controls by Bonferroni's post-hoc test.

## **CSiO<sub>2</sub> exposure leads to increased inflammatory cell infiltration in MARCO<sup>-/-</sup> mice**

Infiltration of immune cells such as AM, DC and neutrophils is an important step in development of pulmonary inflammation following exposure to environmental particles. After CSiO<sub>2</sub> exposure, the results demonstrated an increase (although not significant) in total number of lavaged cells from MARCO<sup>-/-</sup> mice compared to WT mice (Figure 20A). To identify the cell type that was contributing to the increased cellularity shown in figure 20A, lavage cells from CSiO<sub>2</sub> treated MARCO<sup>-/-</sup> and WT mice were stained for cell surface markers to differentiate AM, DC and neutrophils. The majority of the lavage cells were CD11c positive. Additional staining for MHC II revealed two subpopulations; AM (CD11c<sup>+</sup>/ MHC II<sup>lo</sup>) and DC (CD11c<sup>+</sup>/ MHC II<sup>hi</sup>). Since Gr-1 expression is a marker for mature neutrophil, Gr-1 was used to identify neutrophils (CD11b<sup>+</sup>/Gr-1<sup>+</sup>/CD11c<sup>lo</sup>) (Lagasse and Weissman, 1996). Flow cytometric analysis revealed a significant increase in neutrophil infiltration in both WT and MARCO<sup>-/-</sup> mice 24 h post-CSiO<sub>2</sub> exposure (Figure 20B). Furthermore, there was a significant increase in neutrophilia in CSiO<sub>2</sub> treated MARCO<sup>-/-</sup> mice compared with WT CSiO<sub>2</sub> treated mice (Figure 20B). Similarly, only CSiO<sub>2</sub>-treated MARCO<sup>-/-</sup> mice showed significant increases in the numbers of AM and DC as compared to corresponding PBS-treated mice while corresponding WT mice did not (Figure 20 C and D). Taken together, these results further strengthen the observation that there was a marked increase in an inflammatory response in the absence of MARCO in CSiO<sub>2</sub> treated mice.

**A****B****C****D**

**Figure 20: MARCO<sup>-/-</sup> mice show increased inflammatory cell infiltration following CSiO<sub>2</sub> exposure**

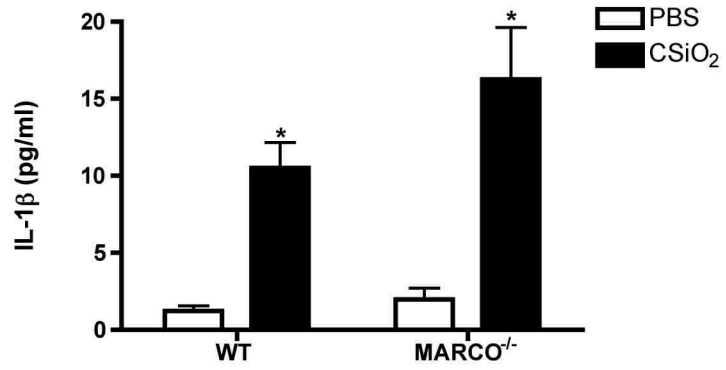
Lavage cells were stained for cell surface markers to differentiate AM, DC and neutrophil populations. **(A)** The total number of lavage cells from both WT and MARCO<sup>-/-</sup> mice increased following CSiO<sub>2</sub> exposure. **(B)** CSiO<sub>2</sub> exposure for 24 h increased recruitment of neutrophils in MARCO<sup>-/-</sup> mice compared with WT mice. Similarly, absence of MARCO also lead to pronounced increases in **(C)** AM and **(D)** DC populations 24 h post-CSiO<sub>2</sub> exposure. The results are represented as mean ± SE. \*p <0.05 compared to PBS treated mice; #, p<0.01 compared to WT CSiO<sub>2</sub> treated mice by Bonferroni's post-hoc test.

### **Increase in levels of early response cytokines following CSiO<sub>2</sub> exposure in MARCO<sup>-/-</sup> mice**

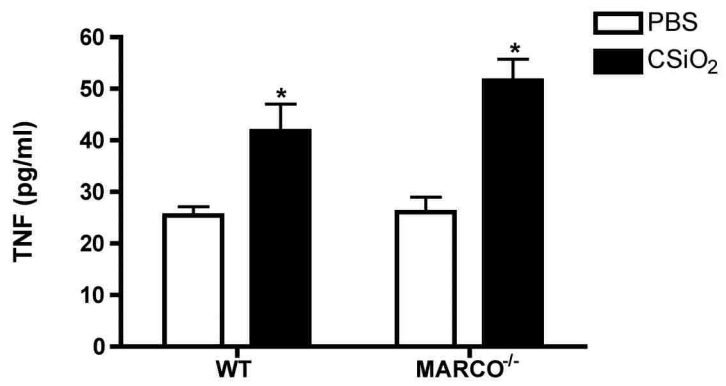
To determine if the induction of lung injury and increased neutrophilia seen in MARCO<sup>-/-</sup> mice following CSiO<sub>2</sub> exposure correlated with increased expression of early response cytokines, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were measured in lavage fluid from PBS and CSiO<sub>2</sub>-treated WT and MARCO<sup>-/-</sup> mice. The levels of all three cytokines were increased in WT and MARCO<sup>-/-</sup> CSiO<sub>2</sub> treated mice compared with the respective PBS controls (Figure 21 A-C). MARCO<sup>-/-</sup> CSiO<sub>2</sub> treated mice exhibited significantly enhanced IL-6 levels following CSiO<sub>2</sub> exposure compared to WT CSiO<sub>2</sub> treated mice (Figure 21 C). The observed differences in cytokine profiles between WT and MARCO<sup>-/-</sup> mice further substantiate the previous results showing increased inflammatory response (Figure 19 and 20) in MARCO<sup>-/-</sup> mice following CSiO<sub>2</sub> exposure.



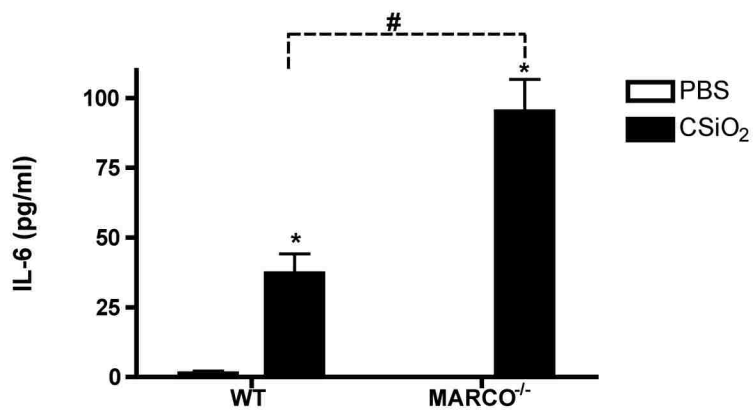
A



B



C



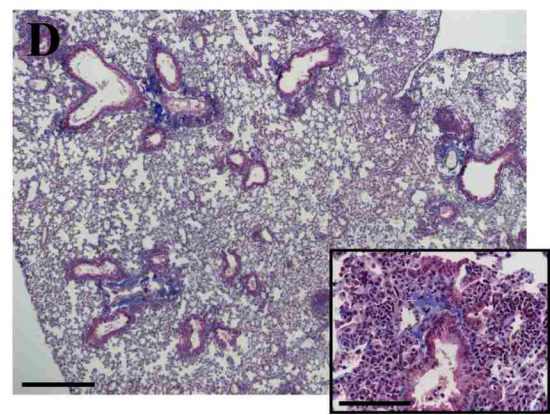
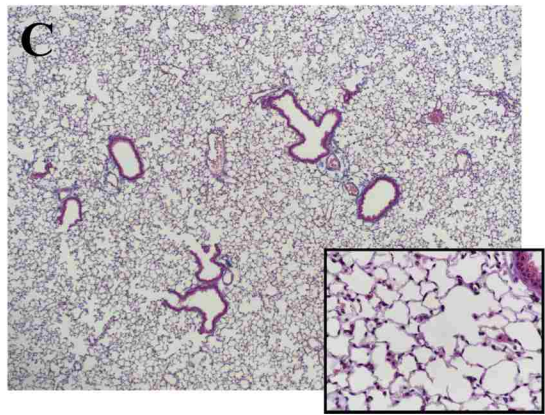
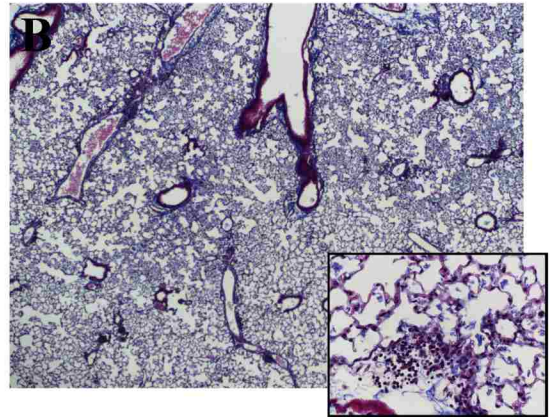
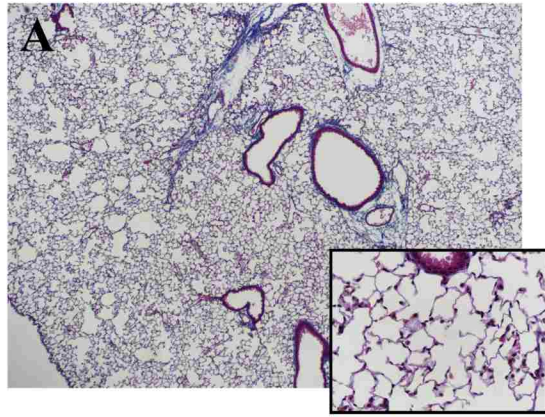
**Figure 21: Increased cytokine response in MARCO<sup>-/-</sup> mice**

After 24 h CSiO<sub>2</sub>-exposure levels of cytokines (A) IL-1 $\beta$  (B) TNF- $\alpha$  and (C) IL-6 were significantly increased in lavage fluid from both WT and MARCO<sup>-/-</sup> mice. A significant increase in IL-6 levels was observed following CSiO<sub>2</sub> exposure in MARCO<sup>-/-</sup> mice compared with WT mice. The results are represented as mean  $\pm$  SE (n=5-6). \* p<0.05, as compared to PBS treated mice. #, p<0.05 compared to CSiO<sub>2</sub>-treated WT mice by Bonferroni's post-hoc test.

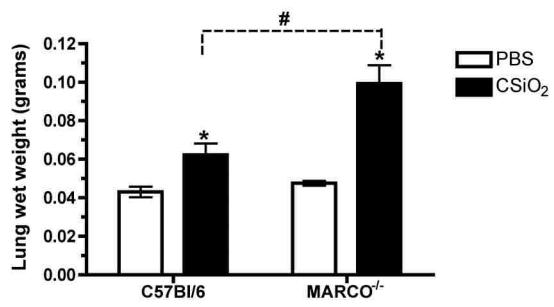
### **Increase in chronic inflammation following CSiO<sub>2</sub> exposure in MARCO<sup>-/-</sup> mice**

Histopathological assessment of lung tissue sections from CSiO<sub>2</sub> exposed mice was performed to assess whether the acute increase in inflammatory mediators correlate with any histopathological changes. WT and MARCO<sup>-/-</sup> mice were instilled with 1 mg of CSiO<sub>2</sub> or 25 µl of PBS, every week for four weeks, three months later the mice were anaesthetized and the lungs were surgically removed and the lung wet weight was assessed and found to be higher in the CSiO<sub>2</sub> exposed MARCO<sup>-/-</sup> mice as compared to PBS treated mice. The difference in lung wet weight following three months, CSiO<sub>2</sub> exposure to WT mice did not reach significance (Figure 22E). The lungs were then fixed and embedded in paraffin blocks. Five micron sections were then analyzed for histological changes (Figure 22 A-D).

Representative sections from PBS treated WT and MARCO<sup>-/-</sup> mice (Figure 22 A and C) showed normal tissue architecture, indicating that the absence of MARCO does not lead to gross anatomical changes. A typical inflammatory response and thickening of interstitium was observed in CSiO<sub>2</sub> treated WT mice (Figure 22B and inset). In comparison, MARCO<sup>-/-</sup> mice demonstrated an increased accumulation of inflammatory cells (Figure 22D and inset). These results indicate that MARCO<sup>-/-</sup> mice show increased chronic inflammation compared to WT mice emphasizing critical role of MARCO in CSiO<sub>2</sub>-induced inflammation.



**E**



**Figure 22: Assessment of lung histopathology following chronic exposure to CSiO<sub>2</sub>.**

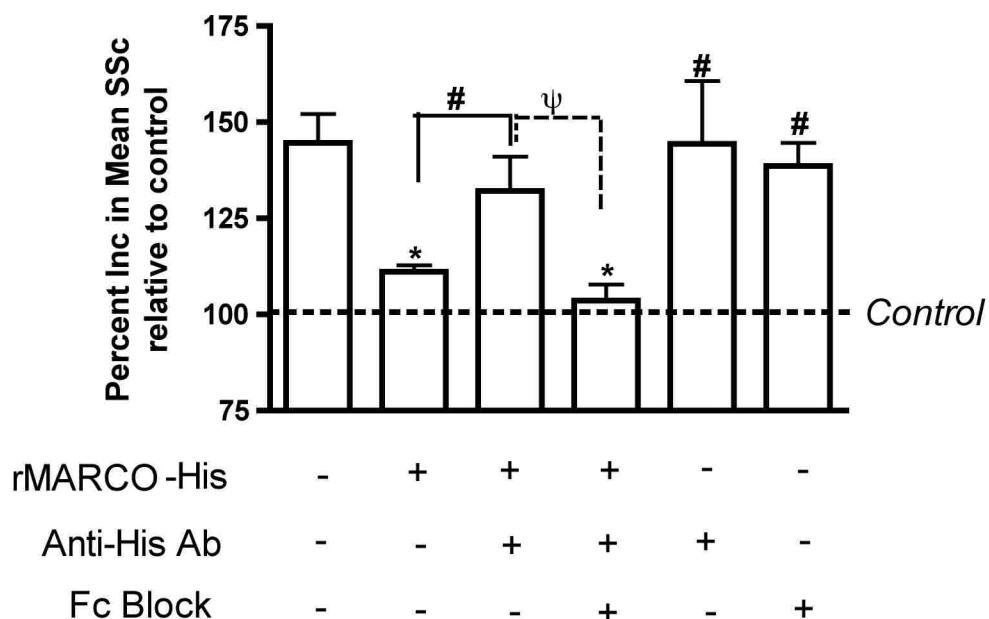
Chronic inflammatory response in the lung was evaluated by gomori's trichrome staining. Representative sections from WT and MARCO<sup>-/-</sup> mice treated with PBS (**A and C**); show normal tissue and cell architecture and no inflammatory response. (**D**) MARCO<sup>-/-</sup> CSiO<sub>2</sub>-exposed mice exhibited enhanced infiltration of inflammatory cells compared with CSiO<sub>2</sub> treated WT mice lung sections (**B**). (Representation of n=5). The magnification bar 25 μm and 12.5 μm (inset). (**E**) The wet lung weight of both WT and MARCO<sup>-/-</sup> mice was significantly increased 3 months following CSiO<sub>2</sub> treatment as compared with PBS treated mice. Results are represented as mean ± SE (n=5-6). \* p<0.05, compared with PBS treated mice; #, p<0.05, compared with CSiO<sub>2</sub> treated MARCO<sup>-/-</sup> mice.

## Facilitating antibody mediated uptake of CSiO<sub>2</sub> particles

Previous studies have shown that efficient uptake and clearance of CSiO<sub>2</sub> particles from the lung is very important for resolution of inflammatory reactions following exposure (Porter *et al.*, 2004). The results from our current report suggest that the absence of MARCO results in decreased CSiO<sub>2</sub> clearance leading to increased neutrophilia, cytokine levels and lung injury. Therefore, increasing the CSiO<sub>2</sub> uptake by AM through an alternative mechanism could help resolve the observed increase in inflammation in MARCO<sup>-/-</sup> mice. As an initial step towards determining feasibility, soluble recombinant MARCO protein was used to evaluate CSiO<sub>2</sub> binding by an alternative pathway.

CSiO<sub>2</sub> particles were coated with recombinant MARCO protein (rMARCO), composed of the extracellular domain of MARCO with C-terminal SRCR domain and a N-terminal nine histidine tag. As expected, rMARCO coated CSiO<sub>2</sub> particles were no longer recognized by the AM, as shown by the significant inhibition of CSiO<sub>2</sub> uptake by AM (Figure 23). Further, treating the coated silica particles with antibody against the N-terminal histidine tag leads to restored recognition and uptake of CSiO<sub>2</sub> particles by AM (Figure 23). In order to test if the Anti his antibody and rMARCO coated CSiO<sub>2</sub> were recognized by Fc receptors on the AM, by binding the Fc portion of the Anti-His antibody, the AM were treated with Fcblock (CD16/CD32) prior to treatment with Anti his-rMARCO-CSiO<sub>2</sub> complex. Indeed, the FcBlock pretreatment completely inhibited the uptake of the CSiO<sub>2</sub> complex. These results support the notion that the Fc portion of the His antibody attached to the rMARCO coated CSiO<sub>2</sub> particles was recognized by the Fc

receptors on the AM. The pretreatment with only Fcblock or the histidine antibody did not affect the uptake of  $\text{CSiO}_2$  particles (Figure 23).



**Figure 23: Antibody mediated uptake of CSiO<sub>2</sub> by AM via Fc Receptors**

Alveolar macrophages (AM) isolated from C57Bl/6 mice showed an increase in side scatter following CSiO<sub>2</sub> (100 µg/ml) treatment (left bar) relative to unexposed control (dotted line). CSiO<sub>2</sub> particles coated with recombinant MARCO (rMARCO) were not recognized by AM (second bar). rMARCO coated CSiO<sub>2</sub> treated with anti His antibody was recognized by the Fc receptors on the AM (third bar), confirmed by FcBlock inhibition of this binding complex (fourth bar). Treatment with FcBlock or anti-his antibody did not lead to inhibition of CSiO<sub>2</sub> binding by AM (last two bars). The results are represented as mean ± SE. \*, p<0.05 compared to uncoated CSiO<sub>2</sub> treatment, #, p<0.05 compared to only rMARCO coated CSiO<sub>2</sub> particles. φ, p<0.05 compared to FcBlock treated AM followed by treatment with rMARCO coated CSiO<sub>2</sub> particles and anti His antibody complex. n=3.



#### 4.4. DISCUSSION

Alveolar macrophages (AM) are the first immune cell type to encounter inhaled CSiO<sub>2</sub> particles (Warheit *et al.*, 1988). Following recognition and uptake of CSiO<sub>2</sub> particles, AM clear some particles from the lung, undergo apoptosis or get activated and secrete various cytokines and growth factors (Lugano *et al.*, 1984). These initial steps contribute to the events leading to fibroblast proliferation and collagen production in the development of silicosis (Bodo *et al.*, 2003; Thakur *et al.*, 2008). An important step following crystalline silica (CSiO<sub>2</sub>) exposure is clearance of CSiO<sub>2</sub> by alveolar macrophages (AM) from the lung through the mucociliary pathway or lymphatic drainage mechanisms (Adamson *et al.*, 1992; Brody *et al.*, 1982). The AM express the scavenger receptor MARCO, which has been identified as a receptor that plays an important role in binding and uptake of CSiO<sub>2</sub> particles in murine models (Hamilton *et al.*, 2006). The purpose of the current study was to analyze the role of MARCO *in vivo*, in CSiO<sub>2</sub> clearance and subsequent inflammation and fibrosis. The results demonstrated that the absence of MARCO resulted in decreased CSiO<sub>2</sub> clearance from lungs, which contributed to increased lung injury and inflammation following CSiO<sub>2</sub> exposure. Furthermore, coating the CSiO<sub>2</sub> particles with rMARCO along with an anti His antibody protein facilitated the uptake of CSiO<sub>2</sub> particles by AM via alternative mechanisms. These studies might have potential therapeutic implications in subjects with diminished ability to clear particles from the lung due to decreased expression or polymorphisms of MARCO.

In the lung, interstitial macrophages (IM) along with AM play a pivotal role in development of silicosis (Migliaccio *et al.*, 2005; Zetterberg *et al.*, 2000). Numerous reports have supported the hypothesis that translocation of CSiO<sub>2</sub> loaded AM from the

alveolar space to the pulmonary interstitium leads to CSiO<sub>2</sub>-IM interaction and enhances activation of the adjacent fibroblasts and development of silicosis (Adamson *et al.*, 1991; Zetterberg *et al.*, 2000). Following three, seven and fourteen days of CSiO<sub>2</sub> exposure the IM from C57Bl/6 and Balb/c mice, showed a significant increase in percentage of MARCO expressing IM (Figure 17A-B). These results are specific to CSiO<sub>2</sub>, because the IM from mice treated with non-fibrogenic particle TiO<sub>2</sub> did not show any increase in MARCO<sup>+</sup> IM (Figure 17A-B). Differences in induction of MARCO expression by CSiO<sub>2</sub> and TiO<sub>2</sub> may be attributed to differential signaling events. In this regard, p38 mitogen activated protein kinases (MAPK) have been reported to play a role in regulation of MARCO (Doyle *et al.*, 2004). Some studies have suggested that AM derived cytokines such as IL-1 $\beta$  are unique to CSiO<sub>2</sub> –induced pulmonary response and TiO<sub>2</sub> exposure does not stimulate their expression (Driscoll *et al.*, 1990; Oghiso and Kubota, 1987). IL-1 $\beta$  could also significantly alter the expression of variety of inflammatory mediators such as cytokines and matrix metalloproteinases and is also known stimulator of p38 MAPK (Feldmann *et al.*, 1996). Therefore it can be speculated that CSiO<sub>2</sub>-induced IL-1 $\beta$  release can induce MARCO expression on the IM. Another possibility is that CSiO<sub>2</sub> directly stimulates IM MARCO expression upon translocation to the interstitial compartment while, TiO<sub>2</sub> does not. CSiO<sub>2</sub> stimulates the p38 MAP kinase pathway (data not shown) consistent with this proposed mechanism of induction of MARCO expression (Ovrevik *et al.*, 2004). Nevertheless, the increased MARCO expression on IM from CSiO<sub>2</sub> exposed mice substantiates the important role of MARCO in immune response against fibrogenic CSiO<sub>2</sub> particles.

Analysis of AM from whole lung lavages of MARCO<sup>-/-</sup> mice showed that MARCO<sup>-/-</sup> AM have significantly diminished ability to bind CSiO<sub>2</sub> particles compared to AM from WT mice (Figure 18A-B). The attenuation of binding and subsequent clearance of CSiO<sub>2</sub> from the lung caused increased microvascular permeability and inflammation in MARCO<sup>-/-</sup> mice as measured by total protein levels in the lavage fluid and increase in lung lavage cellularity, compared to WT mice (Figure 19 and 20A). Furthermore, chronic three-month CSiO<sub>2</sub> exposure in MARCO<sup>-/-</sup> mice led to significant increase in lung wet weight, a marker of persistent inflammation and pulmonary edema (Figure 22E).

An important first step in acute pulmonary inflammatory response to inhaled CSiO<sub>2</sub> particles involves an influx of inflammatory cells (Bowden and Adamson, 1984). Staining the lavage cells for AM, DC and neutrophil cell surface markers following CSiO<sub>2</sub> exposure demonstrated a significant increase in number of all the three immune cells types (Figure 20B-D). After CSiO<sub>2</sub> exposure, there was significant increase in number of neutrophils in MARCO<sup>-/-</sup> mice as compared to WT mice (Figure 20B). Previous studies have reported that another Class A family member SR-A is an important player in CSiO<sub>2</sub> induced inflammatory response. Similar to MARCO<sup>-/-</sup> mice, the SRA<sup>-/-</sup> mice developed enhanced neutrophilia and inflammatory response following CSiO<sub>2</sub> exposure (Beamer and Holian, 2005a). Although the exact role of neutrophils in development of fibrosis is not known, studies suggest that the duration of neutrophil activation correlates with pulmonary fibrosis (Jones *et al.*, 1998).

The CSiO<sub>2</sub> induced inflammatory response is triggered by cytokine release by AM and other immune cells in the lung (Rao *et al.*, 2004; Vanhee *et al.*, 1995). The cytokines and growth factors secreted following CSiO<sub>2</sub> exposure can recruit

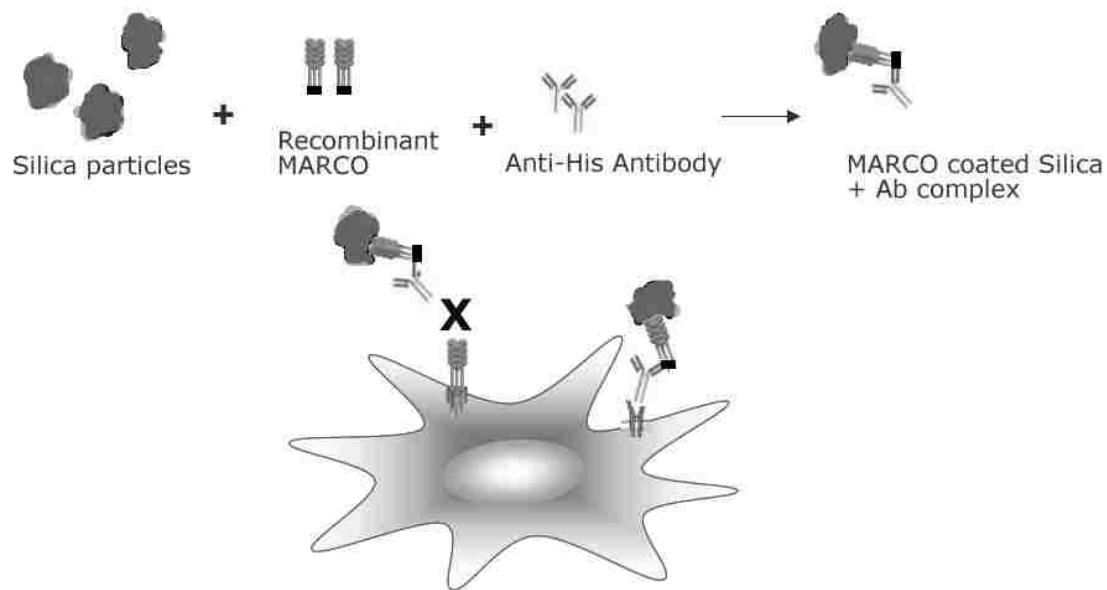
inflammatory cells into the lung and propagate the inflammatory response and lung injury (Hamilton *et al.*, 2008). Among the cytokines secreted, TNF- $\alpha$  and IL-1 $\beta$  have been extensively studied and shown to be important in pathogenesis of CSiO<sub>2</sub>. (Driscoll *et al.*, 1990; Srivastava *et al.*, 2002). In the present study, both TNF- $\alpha$  and IL-1 $\beta$  levels were increased in MARCO<sup>-/-</sup> mice 24 h after CSiO<sub>2</sub> exposure as compared WT mice (Figure 21 A-B). Additionally, IL-6 levels in the lavage fluid from MARCO<sup>-/-</sup> mice was significantly increased 24 h post CSiO<sub>2</sub> instillation as compared to WT mice (Figure 21C). IL-6 is a multifunctional pleiotropic cytokine secreted by immune and non-immune cells. It has numerous physiological functions in acute phase response as well as chronic inflammation (Hodge *et al.*, 2005). These results demonstrate that MARCO<sup>-/-</sup> mice show exacerbated Th1 and Th2 response following CSiO<sub>2</sub> exposure. Furthermore, it has been reported that IL-1 $\beta$ , TNF- $\alpha$  and IL-6 can mediate inflammatory pathology in many autoimmune diseases, and antibodies or receptor antagonists of these inflammatory cytokines are effective therapeutics. (Chatzantoni and Mouzaki, 2006; Ishihara and Hirano, 2002). In this regard, MARCO<sup>-/-</sup> mice have been reported to show increased response to self and antigens and have increased risk of development of autoimmune disease (Wermeling *et al.*, 2007).

The above results demonstrated that the absence of MARCO increases the acute response to CSiO<sub>2</sub> exposure. The effect on the chronic response is also important to determine. In fact, the increased inflammatory response observed in MARCO<sup>-/-</sup> mice following acute (24 h) exposure to CSiO<sub>2</sub> correlated with an increase chronic inflammatory response as evidenced by changes in wet weight (Figure 22E) and histopathological changes three months post-instillation (Figure 22 B and D). Taken

together, these studies support the notion that MARCO is critical for CSiO<sub>2</sub> clearance, as well as acute and chronic pulmonary inflammation.

Most of the current information on the role of MARCO comes from studies using murine models with little information from humans about variability of MARCO expression or association with lung inflammation or fibrosis. MARCO has been reported to be expressed on human AM (Arredouani *et al.*, 2005) and polymorphisms in MARCO might exist. The SRCR domain of human MARCO has been shown to bind CSiO<sub>2</sub> particles avidly (previous chapter). We hypothesized that using the CSiO<sub>2</sub> binding property of MARCO it might be possible to facilitate uptake of CSiO<sub>2</sub> particles using a combination of recombinant MARCO with an antibody against the recombinant MARCO through an alternative pathway or receptor. By coating the CSiO<sub>2</sub> with recombinant MARCO protein (rMARCO) we rendered the CSiO<sub>2</sub> particles unrecognizable by MARCO expressed on AM from healthy WT mice (Figure 23). As a result, we created an in vitro model similar to AM deficient in MARCO expression or expressing aberrant forms of MARCO protein and hence deficient and uptake and clearance of CSiO<sub>2</sub> particles (Figure 23). Subsequently, the rMARCO coated CSiO<sub>2</sub> particles were treated with anti- His tag antibody creating an antigen-antibody complex. There was a significant increase in uptake of CSiO<sub>2</sub>, rMARCO and anti His antibody complex that was blocked by pretreating the cells with FcBlock (Figure 23) highlighting that the complex was now bound by Fc receptors on the AM by recognizing the Fc portion of anti-His antibody complex (Figure 24). Pretreatment of CSiO<sub>2</sub> particles with only anti-His tag antibody or FcBlock did not inhibit the binding of CSiO<sub>2</sub> particles.

In summary, this study lead to some key findings, first, MARCO expression was unregulated in interstitial macrophages following CSiO<sub>2</sub> exposure. Second, MARCO plays a role in clearance of CSiO<sub>2</sub> particles from the lung. Third, MARCO<sup>-/-</sup> mice exhibit increased acute and chronic inflammation following CSiO<sub>2</sub> exposure and lastly, facilitating uptake of CSiO<sub>2</sub> particles by an alternative route might have therapeutic implications in subjects having diminished clearance mechanisms. Together, these findings provide evidence of an important role of MARCO in regulation of inflammation response in vivo.



**Figure 24: Schematic representation of proposed model for antibody mediated CSiO<sub>2</sub> clearance.**

#### 4.5. REFERENCES

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## APPENDIX A

### PRELIMINARY DATA ANALYZING MECHANISTIC ROLE OF MARCO IN CRYSTALLINE SILICA-INDUCED APOPTOTIC SIGNALING

#### 5.1. INTRODUCTION

Free crystalline silica (CSiO<sub>2</sub>), is one of the most common minerals found in the earth's crust (Lalmanach *et al.*, 2006; Rosenman *et al.*, 2003). It is found in sand, rocks such as granite, sandstone and metal quarries. Respirable CSiO<sub>2</sub> is an ubiquitous environmental and occupational fibrogenic agent capable of inducing inflammation, fibroblast proliferation and excess collagen production resulting in lung fibrosis called silicosis (Craighead *et al.*, 1988; Green and Vallyathan, 1996). Silicosis is a prevalent health problem throughout the world particularly in the developing nations (Craighead *et al.*, 1988; Green and Vallyathan, 1996).

Basic and clinical scientists have extensively studied silicosis and yet little is known about the crucial molecular mechanisms that initiate and propagate the process of injury, inflammation and scarring. Although the pathophysiological mechanisms remain unclear, it has been established that the lung responds to CSiO<sub>2</sub> by massive enrollment of AM and other immune cells and triggering an inflammatory cascade of reactions (Lalmanach *et al.*, 2006; Schmidt *et al.*, 1984).

Alveolar macrophages (AM) are primarily responsible for binding, ingestion and ultimately clearance of inhaled particulate matter (Chen and Shi, 2002 May-Jun; Srivastava *et al.*, 2002 Feb). Upon inhalation of CSiO<sub>2</sub> particles, AM engulf CSiO<sub>2</sub> and undergo apoptosis. These apoptotic bodies and free CSiO<sub>2</sub> are engulfed by other AM,

which either secrete proinflammatory cytokines or undergo apoptosis. This cycle of engulfment, apoptosis and cytokine secretion continually lead to prolonged inflammation and is considered to be important in the development of fibrosis (Rimal *et al.*, 2005 Mar; Srivastava *et al.*, 2002 Feb). Previous studies in our laboratory have shown that the scavenger receptor, MARCO plays a vital role in CSiO<sub>2</sub> binding and apoptosis in AM from C57Bl/6 mice (Hamilton *et al.*, 2006). However, mechanistic details of role of MARCO in CSiO<sub>2</sub> induced apoptosis remain elusive. Previously reported observation that absence of MARCO prevents CSiO<sub>2</sub> binding and apoptosis, suggests a mechanistic role of MARCO in the process (Hamilton *et al.*, 2006).

Aggregation of receptors upon binding to specific ligands has been reported in case of a large number of receptors including the TCR/CD3 complex, CD40, TNF-R (Boniface *et al.*, 1998; Chan *et al.*, 2000; Vidalain *et al.*, 2000). Ligand induced receptor clustering results in trans-activation of receptor-associated signaling molecules. This further induces the local assembly of the signaling elements that transmit the extracellular signals into the cell. Most of the known ligands for MARCO such as CSiO<sub>2</sub>, AcLDL are relatively much larger in size than MARCO and it can be speculated that MARCO ligands can trigger clustering of MARCO facilitating enabling binding and uptake of large ligands and particles. Further, studies have demonstrated that ligand binding to receptor leads to activation of acid sphingomyelinase (ASM) an enzyme that catalyzes the conversion of membrane sphingomyelin to ceramide, resulting in release of ceramide in outer leaflet of the cell membrane (Cremesti *et al.*, 2001; Grassme *et al.*, 2003; Grassme *et al.*, 2001b; Grassme *et al.*, 2001a).

Ceramide is an endogenous sphingolipid and acts as second messenger in ASM

mediated apoptosis (Kolesnick and Golde, 1994; Obeid *et al.*, 1993). Various ligands and environmental toxins such as TNF and lipopolysaccharide (LPS) utilize ceramide to induce apoptosis in the target cells (Thomas *et al.*, 2000; Zhang *et al.*, 2001). Further, some of the downstream signaling events in the apoptotic sphingomyelin pathway involve ceramide induced mitochondrial dysfunction, and activation of mitogen activated protein kinases (MAPK) such as SAPK/JNK and p38 (Kong *et al.*, 2005; Xia *et al.*, 1995).

Preliminary studies in our laboratory have also shown that ASM inhibitors; imipramine and desipramine partially block CSiO<sub>2</sub> induced apoptosis in AM. Based on the observation that MARCO is an important receptor for CSiO<sub>2</sub> binding and uptake (Hamilton *et al.*, 2006), we hypothesized that the binding of CSiO<sub>2</sub> to MARCO might lead to activation of ASM and generation of ceramide leading to apoptosis. The present study investigated the role of ASM in MARCO mediated apoptosis using two in vitro models, CHO cells transfected with human MARCO and murine bone marrow derived macrophages.

## **5.2. MATERIALS AND METHODS**

### **Mice**

Breeding pairs of C57Bl/6 mice were originally purchased from The Jackson Laboratory (Bar harbor, ME, USA). All mice were maintained in the University of Montana specific pathogen-free (SPF) laboratory animal facility. The mice were maintained on an ovalbumin-free diet and given deionized water *ad libitum*. The

University of Montana Institutional Animal Care and Use Committee (IACUC) approved all animal procedures.

### **Cell Culture**

Chinese Hamster Ovary (CHO) cells (American Type Culture Collection, Manassas, VA) were cultured in HAM's F-12 medium with 2 mM L-glutamine (Mediatech Inc., Herdon, VA) containing 10% heat-inactivated fetal bovine serum (FBS), 100 IU penicillin, 100 µg/ml streptomycin (Mediatech Inc). CHO cells were transiently transfected with pcDNA 3.1 (E), full length human MARCO (M). All transfections were conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as per manufacturer's instructions. For experiments, transiently transfected CHO cells were gently harvested 24-30 h post-transfection using trypsin-EDTA. Transfection efficiency of the full length MARCO was determined to be 30-40 % by staining the cells for MARCO expression using human MARCO specific antibody (PLK-1) and isotype control followed by treatment of FITC conjugated secondary antibody. Analysis was done using FACS Aria flow cytometer using Diva Software (version 4.1.2; BD Biosciences). All the experiments were conducted within 24-30 h following transient transfections.

### **Bone marrow derived macrophages (BMDMs)**

Bone marrow was aspirated from femurs and tibiae of C57Bl/6 mice (6-10 weeks) using a 3 ml syringe with RPMI culture media supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU penicillin, 100 µg/ml streptomycin (Mediatech Inc.). Following overnight stromal elimination,  $15 \times 10^6$  non-adherent cells were transferred to new flasks, supplemented with murine recombinant macrophage colony-stimulating



factor; final concentration 10 ng/ml (R & D Systems, Minneapolis, MN, USA). Following 4 and 7 days, the media was replenished with MCSF (concentration 5 ng/ml). The cells were allowed to differentiate into macrophages for 10 days before use and the adherent cells were gently scraped and stained for macrophage cell surface markers (CD11b and F480) and non-adherent cells were discarded. Cell viability was determined to be >90 % by trypan blue exclusion before treatments.

### **Particle**

Crystalline silica (CSiO<sub>2</sub>) or Min-U-Sil-5 obtained from Pennsylvania Sand Glass Corporation (Pittsburg, PA) was acid washed in 1 M HCl at 100°C, to remove metals and microbial contamination. The CSiO<sub>2</sub> particles were then washed three times with sterile water and dried at 200°C to remove all water. The stock suspension of CSiO<sub>2</sub> in media was dispersed by sonic disruption for 1 min before each experiment.

### **Cytotoxicity assays**

Transfected CHO cells were harvested as described above and  $1 \times 10^5$  were plated in 96-well plates and treated with CSiO<sub>2</sub> (equivalent to 25 or 50  $\mu\text{g}/\text{cm}^2$ ) for 24 h at 37°C. For experiments using BMDM, the cells were gently harvested as described above and  $1 \times 10^6$  cells were treated with or without 2  $\mu\text{g}/\text{ml}$  of MARCO Antibody (ED31; Serotec, Raleigh, NC, USA) for 20 min at 37°C in suspension culture followed by treatment with 200  $\mu\text{g}/\text{ml}$  of CSiO<sub>2</sub> for 15 min at 37°C in suspension. These CSiO<sub>2</sub> treated BMDM were then plated in 96-well for 4 h. The cell viability of CHO cells and BMDM were analyzed using Cell Titer Blue assay (Promega, Madison, WI) following indicated incubations with CSiO<sub>2</sub>. The Cell titer blue assay is based on the ability of living cells to convert a

redox dye (reazurin) into a fluorescent end product (resorufin). Loss of cell viability is accompanied with loss in metabolizing capacity of cells therefore non-viable cells do not convert reazurin to resorufin, which can be detected using spectrofluorometer. After the incubation step, data were recorded using a plate-reading fluorometer.

### **Cell surface expression of MARCO on BMDM**

Fully differentiated BMDM, were harvested gently from T-75 using tissue-culture flasks as described above and cultured with or without LPS (10 ng/ml), IFN- $\gamma$  (12 ng/ml) and IL-4 (10 ng/ml) in presence of MCSF (10 ng/ml). Cells, ( $1 \times 10^5$ ) were incubated with FcBlock, rat anti-mouse CD16/CD32 (BD Pharmingen, San Jose, CA) diluted 1:100 PBS containing 0.01% sodium azide and 1 % FBS buffer (PAB) for 20 minutes on ice. One microgram of MARCO FITC was added to the cells and incubated at room temperature for one hour. One microgram of macrophage cell surface markers, CD11b PerCP Cy5.5 and F480 APC were added and incubated on ice for 30 min. Cells were washed with PAB and analyzed immediately. Analysis was done using FACS Aria flow cytometer using Diva Software (version 4.1.2; BD Biosciences). Live-dead cell discrimination was done using Hoechst 33258 (Invitrogen, Carlsbad, CA). Compensation of the spectral overlap for each fluorochrome was calculated using anti-rat/ hamster Ig compensation beads (BD Biosciences).

### **Assay for acid sphingomyelinase activity**

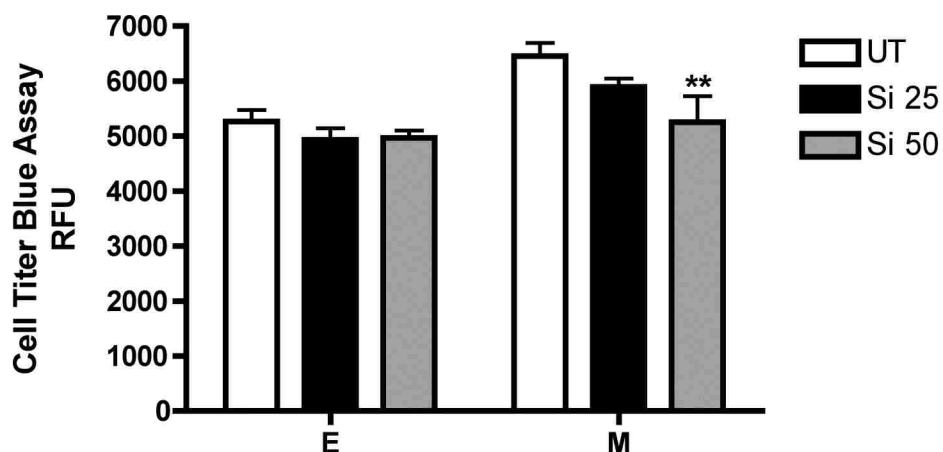
Transfected CHO cells and BMDM were harvested as described above and  $1 \times 10^6$  were treated with  $\text{CSiO}_2$  equivalent to  $50 \mu\text{g}/\text{cm}^2$  and  $200 \mu\text{g}/\text{ml}$  for 30 min at  $37^\circ\text{C}$ , respectively in tumbling suspension culture. The cells were lysed using a cell lysis buffer

containing 1 % Triton X-100 and protease inhibitors (pH 7.4). The lysates were then diluted (1:2) using 50 mM sodium acetate buffer (pH 5.5) and analyzed for acid sphingomyelinase (ASM) activity using the Amplex red sphingomyelinase assay kit (Invitrogen, Carlsbad, CA). In this enzyme-coupled assay, sphingomyelinase activity is monitored indirectly using amplex red reagent, a sensitive fluorogenic probe for H<sub>2</sub>O<sub>2</sub>. First, sphingomyelinase hydrolyses the sphingomyelin to yield ceramide and phosphorylcholine. Phosphorylcholine is then hydrolyzed by alkaline phosphatase to choline. Choline is oxidized by choline oxidase to betaine and H<sub>2</sub>O<sub>2</sub>. Finally, H<sub>2</sub>O<sub>2</sub>, in the presence of horseradish peroxidase, reacts with amplex red reagent in a 1:1 stoichiometry to generate the fluorescent product, resorufin, which was measured by a plate-reading fluorometer. Positive controls included reaction mixture containing sphingomyelinase or 20 mM H<sub>2</sub>O<sub>2</sub> solution or BMDM treated with LPS 10 ng/ml.

### **5.3. RESULTS**

#### **CSiO<sub>2</sub> induced loss of cell viability in MARCO transfected CHO cells**

To investigate whether human MARCO expression in CHO cells increases susceptibility to CSiO<sub>2</sub> induced cytotoxicity, transfected CHO cells were treated with varying concentrations of CSiO<sub>2</sub> for 24 h in 96-well culture plates. The cell titer blue assay revealed increased loss of cell viability in response to CSiO<sub>2</sub> in MARCO transfected CHO cells as compared to empty vector transfected CHO cells (Figure 25). Although, there was a dose response effect of CSiO<sub>2</sub> on decreased cell viability in MARCO transfected cells only 50 µg/cm<sup>2</sup> dose showed a statistically significant loss of viability in MARCO transfected cells. The empty vector transfected cells did not show loss in cell viability following CSiO<sub>2</sub> exposure. These results were consistent with the observation that MARCO transfected CHO cells undergo significant loss in cell viability following CSiO<sub>2</sub> exposure (Chapter 1, page 54).

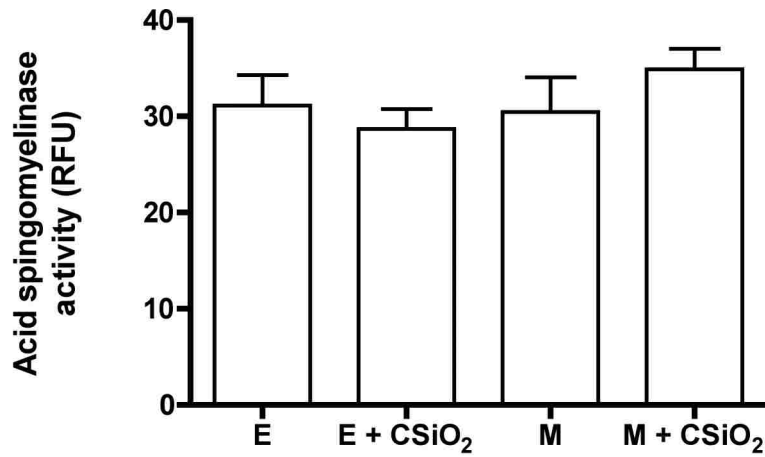


**Figure 25: Crystalline silica induced loss of cell viability in MARCO transfected CHO cells**

Following 24 h of exposure to 25 or 50  $\mu\text{g}/\text{cm}^2$  of  $\text{CSiO}_2$  there is a dose-dependent decrease in cell viability in MARCO transfected (M) cells that reaches significance at 50  $\mu\text{g}/\text{cm}^2$ . Empty vector transfected (E) control show no difference in viability. Results represent Mean  $\pm$  SE, n=3. \*\*, p<0.01, compared to untreated MARCO transfected cells by Bonferroni's post hoc test.

### **CSiO<sub>2</sub>- induced acid sphingomyelinase activity in CHO cells**

Previous work from our laboratory showed that inhibitors of acid sphingomyelinase (ASM), desipramine and imipramine attenuate CSiO<sub>2</sub>-induced apoptosis of alveolar macrophages (AM). To assess whether ASM is activated following CSiO<sub>2</sub> exposure, empty vector and MARCO transfected CHO cells were treated with CSiO<sub>2</sub> for 30 min in 6-well plates. Results from amplex red sphingomyelinase assay demonstrated that there was no increase in ASM activity in lysates from CSiO<sub>2</sub> treated MARCO expressing CHO cells (Figure 26). These findings suggest that the apoptotic sphingomyelin pathway involving ASM induced ceramide generation may not be important in CSiO<sub>2</sub>-induced and MARCO mediated apoptosis. Alternatively, another possibility is that human MARCO transfected CHO cells May not be a good model for measuring CSiO<sub>2</sub> induced ASM activity, since ASM mediated apoptosis is an apoptotic mechanism mainly found in macrophages (Falcone *et al.*, 2004; Wang *et al.*, 2007).



**Figure 26: CSiO<sub>2</sub> does not increase acid sphingomyelinase activity in MARCO transfected CHO cells.**

Empty vector and MARCO transfected CHO cells were treated CSiO<sub>2</sub> (200 µg/ml) for 30 min and lysed in an acidic cell lysis buffer. Analyzing the ASM activity in the lysates demonstrated that CSiO<sub>2</sub> did not increase ASM activity in both control and MARCO transfected cells. Results represent mean ± SE. Sample size n=3.

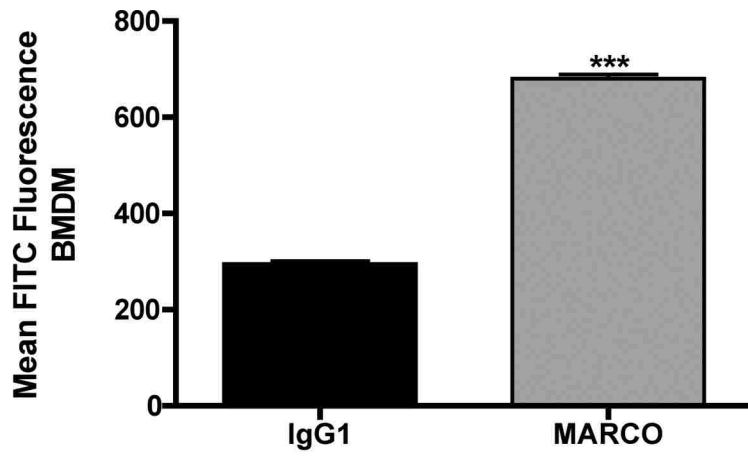
## **MARCO expression on BMDM**

To study the role of MARCO in CSiO<sub>2</sub> induced apoptosis in more relevant cellular model, bone marrow derived macrophages (BMDM) from C57Bl/6 mice were utilized. Murine BMDM are fully differentiated macrophages developed using mononuclear phagocyte precursor cells from the bone marrow in the presence of macrophage colony stimulating factor (MCSF) (Pfau *et al.*, 2004b). Unlike primary macrophages, the BMDM can be maintained in culture for a longer time and would be a convenient model to study CSiO<sub>2</sub> induced MARCO mediated signaling mechanisms.

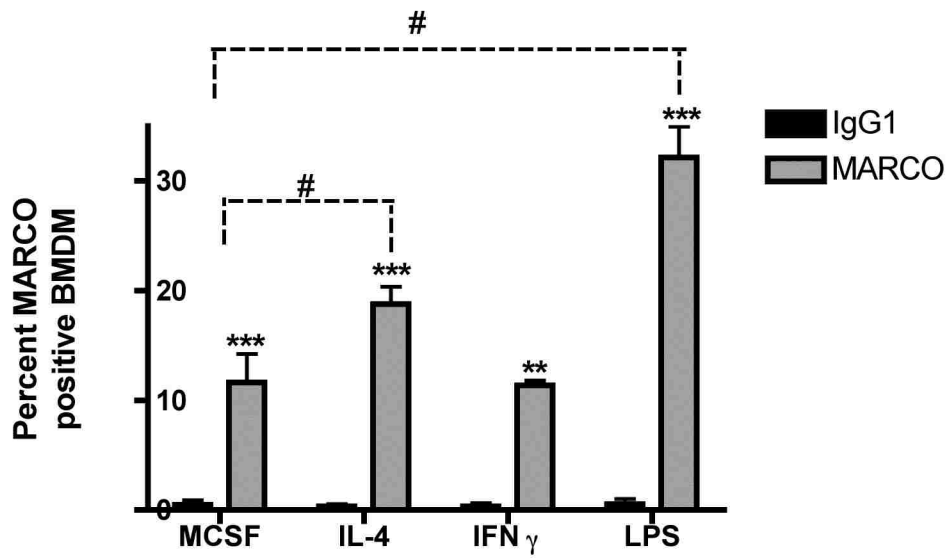
To further validate the use of BMDM from C57Bl/6 mice, we analyzed the cell surface expression of MARCO on differentiated BMDM. Flow cytometric analysis revealed that fully differentiated BMDM expressed MARCO as indicated by the significant increase in mean channel fluorescence in MARCO antibody treated BMDM as compare to isotype treated controls (Figure 27A). In order to analyze the effect of various cytokines and antigens on MARCO expression and create a more efficient model for studying the role of MARCO in CSiO<sub>2</sub> induced macrophage apoptosis, the BMDM were stimulated with IL-4, IFN $\gamma$  or as a positive control, LPS (Meng *et al.*, 2006). MCSF differentiated BMDM show slight, but a significant increase following IL-4 stimulation, while IFN $\gamma$  did not induce any increase in MARCO expression over basal values (Figure 27 B). As expected, LPS was a potent inducer of MARCO expression (Figure 27B). However, LPS stimulated BMDM could not be used as a model for measuring CSiO<sub>2</sub> induced ASM activity, because LPS is known to be a stimulator of ASM in macrophages (Sakata *et al.*, 2007).



A



B



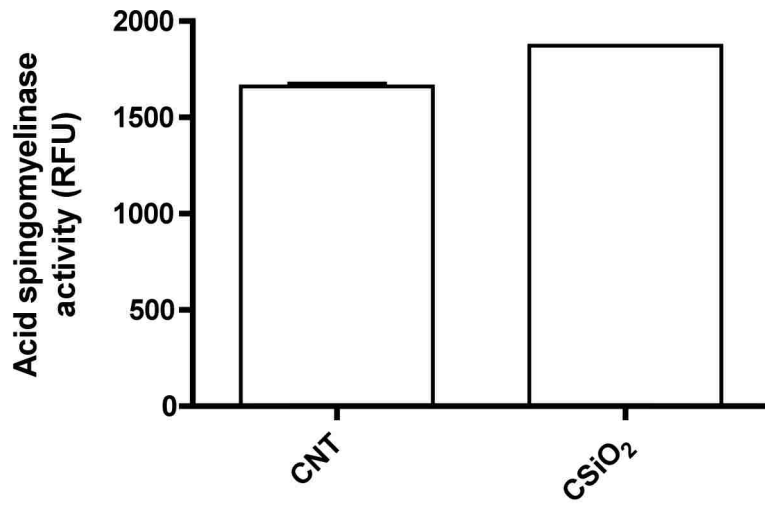
**Figure 27: Regulation of MARCO expression on BMDM**

(A) C57Bl/6 BMDM stained with MARCO-specific antibody, ED31 showed increase in mean fluorescence intensity as compared to the isotype treated controls. (B) BMDM differentiated in presence of MCSF were stimulated overnight with IL-4 (10 ng/ml), IFN $\gamma$  (12 ng/ml) and LPS (10 ng/ml, positive control). Significant increases in the percent cells expressing MARCO was observed following IL-4 treatment, but not with IFN $\gamma$ . As expected, LPS treatment leads to significant increase in MARCO expression. \*\*\*  $p < 0.001$ , compared to controls by Bonferroni's post-hoc test.  $n = 3$ .

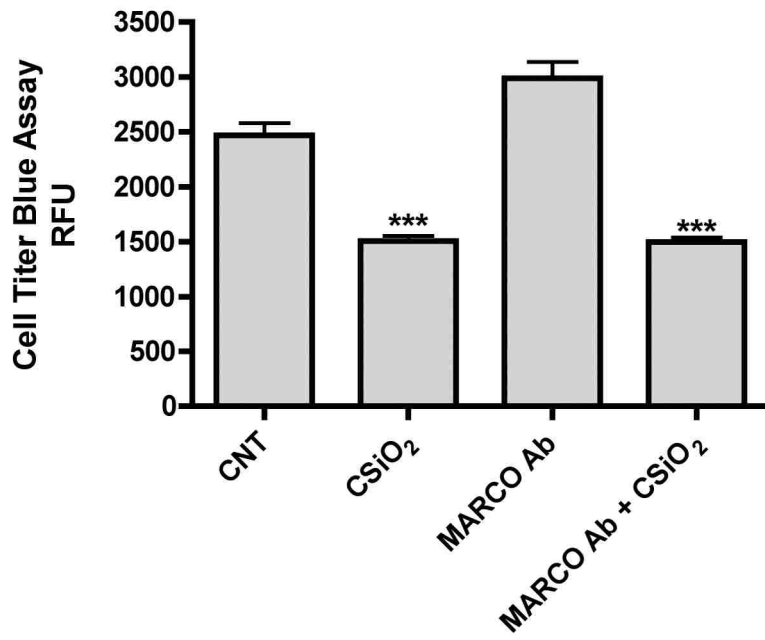
### **Effect of CSiO<sub>2</sub> treatment on acid sphingomyelinase activity in BMDM**

Since LPS stimulated BMDM would prove a complicated model to study CSiO<sub>2</sub> induced signaling mechanisms and the other cytokine stimulations did not lead to a useful increase in MARCO expression, the signaling studies were performed using unstimulated BMDM. In order to test the hypothesis that CSiO<sub>2</sub> exposure leads to an increase in ASM activity, which may further contribute to increase in ceramide levels and subsequent cytotoxicity, BMDM for C57Bl/6 mice were treated with or without CSiO<sub>2</sub> for 30 min and the ASM activity was measured in the lysates. Similar to transfected CHO cells, no significant increase in ASM activity was observed following CSiO<sub>2</sub> exposure in the murine BMDM model (Figure 28A). Unfortunately, the results are not surprising considering the fact that only 10-15 % of MCSF differentiated BMDM show MARCO expression. This observation was further supported with the results showing that pretreatment of BMDM with MARCO antibody does not lead to inhibition of CSiO<sub>2</sub> mediated loss in cell viability measured by cell titer blue assay 4 h following exposure (Figure 28B). Taken together, the results could not be used to test the role of that the sphingomyelin pathway of apoptosis in CSiO<sub>2</sub> induced macrophage apoptosis. In order to elucidate MARCO mediated signaling events in response to CSiO<sub>2</sub>, an appropriate cell model such as immortalized primary cells from C57Bl/6 and MARCO<sup>-/-</sup> mice or a cell line, which constitutively expresses high level of MARCO needs to be used.

**A**



**B**



**Figure 28: Role of MARCO in CSiO<sub>2</sub> induced macrophage apoptosis**

(A) CSiO<sub>2</sub> treatment for 30 min did not lead to an increase in ASM activity in BMDM as compared to control (B) MARCO antibody pretreatment did not inhibit CSiO<sub>2</sub> induced loss of cell viability. Results represent mean  $\pm$  SE. \*\*\*,  $p < 0.001$ , compared to untreated and only MARCO antibody treated controls.  $n=3$ .

## 5.4. CONCLUSION

Scavenger receptor MARCO has been reported to be an important alveolar macrophage (AM) receptor in crystalline silica (CSiO<sub>2</sub>) uptake and cytotoxicity (Hamilton *et al.*, 2006). Although the role of MARCO in CSiO<sub>2</sub>-induced cytotoxicity has been reported, the signaling molecules activated following CSiO<sub>2</sub> binding to MARCO are as yet unknown (Hamilton *et al.*, 2006). Activation of acid sphingomyelinase (ASM), an enzyme responsible for producing a pro-apoptotic second messenger ceramide has been shown to promote macrophage apoptosis following exposure to various environmental challenges such as bacteria, lipopolysaccharide and UV radiation (Kolesnick and Golde, 1994). Previous literature reports and studies in our laboratory have shown that imipramine and desipramine: inhibitors of ASM partially block CSiO<sub>2</sub>-induced apoptosis in AM (unpublished data) and MH-S cells (Thibodeau *et al.*, 2003). The primary objective of this study was to understand the role of ASM in apoptosis induced following MARCO-CSiO<sub>2</sub> interaction. The present study suggests that activation of ASM may not be an early event in CSiO<sub>2</sub>-induced apoptosis in C57Bl/6 BMDM. Additionally, the current study further substantiates the need for development of appropriate cell models to study the signaling events triggered following CSiO<sub>2</sub>-MARCO interactions.

Macrophages express a variety of pattern recognition receptors such as TLR, different members of scavenger receptors, C-type lectins and  $\beta$ 2-integrins. The presence of wide range of pattern recognition receptors with overlapping functions on macrophages makes it difficult to study signaling events triggered following CSiO<sub>2</sub>-MARCO interaction. An *in vitro* cell model containing CHO cells transfected with MARCO seemed to be a good cellular model to study the MARCO mediated signaling

mechanism. Since, the number of pattern recognition receptors that might have overlapping binding properties with MARCO are absent in CHO cells. In accordance to role of MARCO in CSiO<sub>2</sub> binding and cytotoxicity (Hamilton *et al.*, 2006), studies conducted in MARCO transfected CHO cells showed that 24 h treatment with CSiO<sub>2</sub> lead to a dose-dependent loss in cell viability as compared to empty vector transfected controls (Figure 25).

The sphingomyelin pathway of apoptosis involves stimuli-driven activation of ASM, which, further increases ceramide concentration in the cells and triggers the apoptotic machinery. It has been reported that ASM inhibitors significantly prevent cell death in a macrophage cell line in response to CSiO<sub>2</sub> (Thibodeau *et al.*, 2003). Concurrently, previous studies from our laboratory show that ASM inhibitors attenuate CSiO<sub>2</sub>-induced AM apoptosis. Since MARCO is a predominant receptor for CSiO<sub>2</sub> binding (Chapter 1 and 2), in order to determine the role of MARCO-CSiO<sub>2</sub> interaction in ASM activation, the lysates from CSiO<sub>2</sub> treated MARCO expressing CHO cells were analyzed for ASM activity. The results indicated that MARCO transfected CHO cells did not show an increase in ASM activity following CSiO<sub>2</sub> exposure (Figure 26). These results suggested that in contrast to AM, ASM is not an essential component of CSiO<sub>2</sub>-induced apoptosis in CHO cells. Therefore, further studies were conducted using bone marrow derived macrophages (BMDM) from C57Bl/6 mice, which have being previously found to be phenotypically similar to AM (Migliaccio *et.al.*, 2005).

To examine the suitability of murine BMDM, as a system for studying role of MARCO in CSiO<sub>2</sub> induced cytotoxicity, the expression of MARCO on C57Bl/6 BMDM was analyzed. Staining the BMDM for MARCO expression revealed that only 10-15% of

BMDM express MARCO constitutively (Figure 27B). In order to increase the MARCO expression and create a better model for mechanistic studies, the BMDM were cultured with cytokines such as IFN $\gamma$  and IL-4, while LPS was used as positive control. The different proinflammatory cytokines had little or no effect on MARCO expression. IL-4 induced slight but significant increase in the MARCO expression on BMDM while, IFN $\gamma$  had no effect (Figure 27B). As expected, LPS had the greatest effect on induction of MARCO expression (Figure 27B). Despite the strong effect of LPS on MARCO expression, BMDM stimulated with LPS could not be used for studying CSiO<sub>2</sub> induced apoptosis, as LPS alone is known to stimulate ASM activity in macrophages (Sakata *et al.*, 2007). In the light of above observations, the unstimulated BMDM were used to investigate the role of sphingomyelin pathway in CSiO<sub>2</sub>-induced apoptotic signaling. Pretreating C57Bl/6 BMDM with MARCO specific antibody did not inhibit CSiO<sub>2</sub>-induced apoptosis (Figure 28B). These results were consistent with the low constitutive expression of MARCO on C57Bl/6 BMDM and do not diminish the importance of MARCO in CSiO<sub>2</sub> binding and cytotoxicity (Figure 27 and Chapter 1). Lack of a good cellular model with abundant constitutive MARCO expression limits the studies of MARCO- mediated signaling events in response to CSiO<sub>2</sub>. Also, the studies are further complicated by relatively significant expression of another family member of MARCO on BMDM; SRA I/II that has been previously shown to bind CSiO<sub>2</sub> particles and is expressed at relatively higher levels on BMDM (data not shown) (Hamilton *et al.*, 2000). Additionally, CSiO<sub>2</sub> particles are also hypothesized to be phagocytosed by a non-receptor mediated pathway (Hamilton *et al.*, 2006).



Similar to MARCO-transfected CHO cells, ASM activity measured in the lysates from control and CSiO<sub>2</sub>-treated BMDM lysates was not unchanged at 30 min following CSiO<sub>2</sub> exposure (Figure 28A). Further studies at different time points need to be examined to analyze the role of ASM in CSiO<sub>2</sub>-induced macrophage apoptosis. An important obstacle in studying the mechanistic role of MARCO in CSiO<sub>2</sub> induced apoptosis is the lack of a macrophage cell line with high level of MARCO expression. Alternative approaches could focus on creating stably transfected macrophage cell lines using viral vectors, since, transfection of macrophage cell line using lipofectamine have not generated a stably transfected cell line to date.

MARCO is expressed at relatively low levels on macrophages in an aseptic environment but the expression is dramatically increased in response to infection or an immunological challenge *in vivo* (Chapter 3, (Dahl *et al.*, 2007). Therefore it is difficult to develop a system that models, MARCO expression patterns and signaling events triggered following MARCO-CSiO<sub>2</sub> interaction. Nevertheless, the results from this study suggest that ASM and hence the sphingomyelin pathway is not important for MARCO-mediated CSiO<sub>2</sub>-induced apoptosis. Further studies using an appropriate cell model with sufficient constitutive expression of MARCO will help confirm the observations from the current study.

## 5.5. REFERENCES

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## SUMMARY

Numerous pulmonary disorders such as silicosis or pulmonary fibrosis, chronic obstructive pulmonary disorders (COPD) as well as systemic immune deficits have been associated with occupational exposure to inhaled crystalline silica (CSiO<sub>2</sub>) (Churchyard *et al.*, 2004; Englert *et al.*, 2000; Madl *et al.*, 2008). Although the anatomical changes following CSiO<sub>2</sub> exposure been well characterized, the molecular mechanisms underlying CSiO<sub>2</sub>-induced disease outcomes are not yet completely understood. Following deposition of CSiO<sub>2</sub> in the respiratory tract, the resident alveolar macrophages (AM) trigger an acute influx of immune cells from the periphery (Bowden, 1987; Driscoll *et al.*, 1990). Like many other lung diseases such as emphysema and asthma, this inflammatory response plays an important role in development of silicosis. Very few studies have focused on directly identifying the AM receptors involved in initial recognition of CSiO<sub>2</sub> and the implications of this receptor-CSiO<sub>2</sub> interaction on subsequent inflammatory response. Therefore, this project mainly focused on identifying MARCO as the AM receptor that recognizes and clears the CSiO<sub>2</sub> particles from the lungs. Further the goals of this project were to identify the particle binding sites of MARCO and to understand the physiological role of MARCO in CSiO<sub>2</sub>-induced immunopathological changes.

Apoptosis or programmed cell death of AM has been shown to be an important player in CSiO<sub>2</sub> induced pulmonary inflammation (Hamilton *et al.*, 2008). A current paradigm in the field is that phagocytosis of CSiO<sub>2</sub> results in AM apoptosis, with subsequent release of intracellular CSiO<sub>2</sub> from dying cells, this results in multiple ingestion-reingestion cycles, which perpetuates the disease process (Rimal *et al.*, 2005). Previous studies from our laboratory have suggested the role of two splice variants of

scavenger receptor A SRA I and II (SRAI/II), in CSiO<sub>2</sub>-induced apoptosis (Beamer and Holian, 2005a; Chao *et al.*, 2001). Studies conducted to determine the relative contribution of SRA I/II and MARCO in CSiO<sub>2</sub> uptake and cytotoxicity showed that MARCO was a predominant receptor responsible for binding of CSiO<sub>2</sub> particles and subsequent cytotoxicity. *In vitro* studies showed that AM from MARCO<sup>-/-</sup> and MARCO<sup>-/-</sup>/SRA I/II<sup>-/-</sup> mice did not undergo loss of cell viability or apoptosis following CSiO<sub>2</sub> exposure when compared to WT AM. Further, the uptake of CSiO<sub>2</sub> by MARCO<sup>-/-</sup> AM *in vitro* was significantly inhibited relative to C57Bl/6 WT AM. Consistent with the results obtained from MARCO<sup>-/-</sup> mice, pretreatment of WT AM with MARCO antibody diminished the CSiO<sub>2</sub> uptake and efficiently blocked the loss of cell viability and apoptosis. In order to confirm the relevance of murine data to humans, an *in vitro* model comprising of CHO cells transfected with human MARCO was developed. As expected, the MARCO transfected cells efficiently bound CSiO<sub>2</sub> as compared with the control empty vector transfected cells. The results from the cytotoxicity assay correlated with the binding studies with only MARCO transfected cells undergoing apoptosis.

Previous reports from our laboratory showed that *in vitro* binding of CSiO<sub>2</sub> by MARCO<sup>+</sup> AM, increases their antigen presenting (APC) activity. Therefore, studies were conducted to investigate the functional consequences of impaired CSiO<sub>2</sub> binding by MARCO<sup>-/-</sup> AM. The changes in APC activity were assessed by measuring AM-induced T cell cytokine release in co-culture experiments (Hamilton *et al.*, 2001; Migliaccio *et al.*, 2005). The results demonstrated that characterized ability of CSiO<sub>2</sub>-treated MARCO<sup>+</sup> AM to stimulate increased T cell cytokine (IL-13, IFN- $\gamma$ ) release in response to antigens

was attenuated in MARCO<sup>-/-</sup> AM. Taken together, these results identified MARCO the predominant receptor for CSiO<sub>2</sub> binding and cytotoxicity.

One of the intriguing observations from this study was that a commonly used control particle titanium dioxide (TiO<sub>2</sub>), which is a chemically similar to CSiO<sub>2</sub>, had no effect on the APC activity of AM. Additionally, it is well accepted that TiO<sub>2</sub> does not cause pulmonary fibrosis. Also, the acute inflammatory response developed following TiO<sub>2</sub> exposure is reversible and does not lead to any systemic effects (Driscoll *et al.*, 1990). Previous reports have identified TiO<sub>2</sub> as a ligand for MARCO (Arredouani *et al.*, 2005). In order to examine the factors that contribute to contrasting outcomes following CSiO<sub>2</sub> and TiO<sub>2</sub> exposure (despite binding to a common receptor MARCO), we analyzed the different factors affecting the binding of these particles to MARCO. We also investigated the role of MARCO in another non-fibrogenic particle, amorphous silica (ASiO<sub>2</sub>) binding and examined the effect the three particles on AM viability. Previous reports and results from current study showed that all three particles bound to MARCO but only CSiO<sub>2</sub> was toxic (Iyer *et.al.*, 1996b).

Various truncated MARCO mutants were used to identify the binding site of CSiO<sub>2</sub>, TiO<sub>2</sub> or ASiO<sub>2</sub> with a notion that differences in the individual particle-binding site might lead to varying conformational changes in MARCO and help explain the differences in signaling events. The results demonstrated that all three tested particles required the entire C-terminal cysteine rich (SRCR) domain of MARCO for binding. Nevertheless competitive binding studies conducted by pretreating MARCO transfected CHO cells with CSiO<sub>2</sub> or TiO<sub>2</sub> demonstrated that only CSiO<sub>2</sub> blocked ASiO<sub>2</sub> binding while, TiO<sub>2</sub> did not. These observations suggest that CSiO<sub>2</sub> and ASiO<sub>2</sub> share a common

binding site while TiO<sub>2</sub> might have subtle binding differences. Given the relatively large size of these particles compared with MARCO, these results are hard to explain however additional mutational analysis of specific amino acids will help detect the subtle differences in binding sites of these particles. Another notable observation was that divalent cations were necessary for TiO<sub>2</sub> binding to MARCO while CSiO<sub>2</sub> and ASiO<sub>2</sub> binding was not. This study brought to light important subtle differences in binding patterns of the toxic CSiO<sub>2</sub> particles and non-toxic ASiO<sub>2</sub> and TiO<sub>2</sub>. These differences may influence the conformational change in MARCO following individual particle binding and hence affect subsequent signaling mechanisms triggered by these particles.

Studies conducted using C57Bl/6 (WT) and MARCO<sup>-/-</sup> mice post-CSiO<sub>2</sub> instillation demonstrated that absence of MARCO enhances both acute and chronic inflammatory response. The MARCO<sup>-/-</sup> mice demonstrated increase in number of AM, DC and more markedly neutrophils one day post CSiO<sub>2</sub> instillation. Comparison of the cytokine levels in the lavage fluid from WT and MARCO<sup>-/-</sup> AM revealed that levels of IL-6 were significantly higher in MARCO<sup>-/-</sup> mice, also, TNF $\alpha$  and IL-1 $\beta$  were increased in MARCO<sup>-/-</sup> mice. These results clearly suggest that MARCO plays a protective role in CSiO<sub>2</sub>-induced pulmonary inflammation. Particle clearance from the lung is considered to be an important step in resolution of particle-induced inflammation (Adamson *et al.*, 1992). In order to test whether inability of MARCO<sup>-/-</sup> AM to bind CSiO<sub>2</sub> leads to impaired clearance of CSiO<sub>2</sub> from the lung, the CSiO<sub>2</sub> uptake following intranasal instillations by AM from WT and MARCO<sup>-/-</sup> mice were compared. As expected MARCO<sup>-/-</sup> mice showed significantly diminished CSiO<sub>2</sub> binding, which led to unsuccessful clearance of CSiO<sub>2</sub> from the lung eventually contributing to increased lung



injury and inflammation. To study the effects of absence of MARCO on chronic inflammation and to explore if any compensatory mechanism attenuates the long term inflammation, lung wet weight and histomorphological analysis was performed three month post-CSiO<sub>2</sub> instillations in WT and MARCO<sup>-/-</sup> mice. The results demonstrated that there was significant increase in wet lung weights of MARCO<sup>-/-</sup> mice and histological analysis showed a persistent increase in inflammatory cell infiltration in MARCO<sup>-/-</sup> mice as compared with WT mice. Therefore, the absence of MARCO leads to decreased CSiO<sub>2</sub> clearance from the lung, which further contributes to persistent increase in subsequent inflammatory response.

Based on the above results we hypothesized that the CSiO<sub>2</sub>-induced inflammation may be reduced if the rate of clearance of CSiO<sub>2</sub> from the lungs is increased (Figure 24). Therefore, we used the ability of MARCO to bind CSiO<sub>2</sub> to enhance the clearance from the lungs. Soluble recombinant MARCO (rMARCO) protein containing the SRCR domain (CSiO<sub>2</sub> binding site) of MARCO was used to coat CSiO<sub>2</sub> particles. C57Bl/6 AM were then incubated with coated and uncoated CSiO<sub>2</sub> particles. As expected the coating of CSiO<sub>2</sub> particles with rMARCO significantly diminished uptake, as the rMARCO coated CSiO<sub>2</sub> was no longer recognized by MARCO<sup>+</sup> AM. In order to increase the recognition of CSiO<sub>2</sub> particles by Fc receptors, the rMARCO coated CSiO<sub>2</sub> particles were then coated with anti-His tag antibody to create a CSiO<sub>2</sub>, rMARCO and anti His antibody complex. This particle-protein complex was avidly bound by the Fc receptors on the AM by recognizing the Fc portion of anti-His antibody. These studies suggest that a chimeric protein with SRCR domain of MARCO and Fc portion of an opsonin may be used to

increase clearance of CSiO<sub>2</sub> particles in subjects showing polymorphisms in MARCO. Future proof-of-concept *in vivo* studies are underway to test this hypothesis

## 6.1. CONCLUSIONS

Using healthy murine (C57Bl/6) AM and human MARCO transfected CHO cells, this project identified scavenger receptor MARCO as a predominant receptor in binding and cytotoxicity of CSiO<sub>2</sub>. Furthermore we identified the SRCR domain of MARCO as the binding site of three inorganic particles CSiO<sub>2</sub>, TiO<sub>2</sub> and ASiO<sub>2</sub>. Additionally, the results indicate that all three tested particles show unique differences in binding requirements such as need for divalent cations, in binding to MARCO. These differences can form basis for the differences observed in the cytotoxic signaling events triggered by each particle.

*In vivo* studies, using MARCO<sup>-/-</sup> mice confirmed the importance of MARCO in CSiO<sub>2</sub>-induced inflammatory response. MARCO<sup>-/-</sup> mice showed increased acute and chronic inflammation in response to CSiO<sub>2</sub>, marked by increased inflammatory cell infiltration, levels of inflammatory cytokines and persistent histomorphological changes as compared with WT (MARCO<sup>+</sup>) mice. *In vivo* binding studies indicated MARCO<sup>-/-</sup> mice exhibit diminished clearance of CSiO<sub>2</sub> particles from the lungs as compared to WT mice. The prolonged contact CSiO<sub>2</sub> particles with the epithelial cells and other immune cells leads to enhanced lung injury and chronic inflammation. Preliminary studies were conducted to increase uptake of CSiO<sub>2</sub> particles by alternative mechanism (Fc Receptors). These studies can further form the basis for increasing the diminished clearance of CSiO<sub>2</sub> particles from MARCO<sup>-/-</sup> mice. The results from this study give a positive direction to use of recombinant protein containing the SRCR domain of MARCO

to be used as therapeutic intervention in ameliorating CSiO<sub>2</sub> induced inflammation and hence diseases such as silicosis and chronic obstructive pulmonary disease (COPD). Taken together, the results from this project establish MARCO as a key receptor in CSiO<sub>2</sub> uptake and inflammation. The results also provide basis for designing chimeric proteins that may prove therapeutically important in CSiO<sub>2</sub> induced disease outcomes.

## **6.2. FUTURE DIRECTIONS**

Fibrogenic potential of a particle has been thought to be closely associated with the ability of the particle to induce apoptosis, particularly in AM. These studies have provided strong evidence for the role of MARCO in CSiO<sub>2</sub>-induced apoptosis. Elucidating the signaling events triggered by MARCO that lead to AM apoptosis will help understand the molecular details of role of MARCO in CSiO<sub>2</sub> induced cytotoxicity and inflammation.

This study examined the differences in binding of three chemically similar but pathologically different inorganic particles to MARCO, however, additional studies with specific amino acid mutations in SRCR domain need to be conducted to determine the amino acids important for binding of each particle. Proteomics studies such as circular dichroism and X-ray crystallography can be conducted to examine the conformational changes in MARCO following binding of CSiO<sub>2</sub>, ASiO<sub>2</sub> and TiO<sub>2</sub>. Also analyzing TiO<sub>2</sub> binding using proteomic techniques in presence and absence of divalent cations which, were found necessary for TiO<sub>2</sub> binding to MARCO, would provide further proof of concept. The ability of MARCO to bind two particles simultaneously (TiO<sub>2</sub> and ASiO<sub>2</sub>) as well as the differences observed in binding requirements of toxic CSiO<sub>2</sub> and nontoxic

TiO<sub>2</sub> raise important questions regarding *in vivo* disease outcomes in case of co-exposure to these harmful particles.

*In vivo* studies demonstrated that absence of MARCO diminished CSiO<sub>2</sub> clearance and consequently enhanced inflammation as compared to CSiO<sub>2</sub> exposed WT mice. Therefore, studying the clearance or measuring the difference in rate of clearance between WT and MARCO<sup>-/-</sup> mice is of importance for understanding the CSiO<sub>2</sub>-induced pulmonary as well as systemic (autoimmune diseases) effects. Tracking clearance of fluorescent CSiO<sub>2</sub> particles from the alveolar space to interstitium and then to lymph nodes using flow cytometry techniques will prove helpful. Additionally, *in vivo* studies analyzing the effect of pretreatment of recombinant MARCO (rMARCO) on inflammation in CSiO<sub>2</sub> treated MARCO<sup>-/-</sup> mice need to be conducted. Further, understanding the mechanistic details of MARCO-CSiO<sub>2</sub> interactions and elucidating the molecular details of MARCO mediated effects on clearance and CSiO<sub>2</sub>-induced inflammation will provide insight into mechanisms of other particulate matter and MARCO ligands in disease processes.

### 6.3. REFERENCES

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