THE ROLE OF GENDER AND SECRETED PHOSPHOPROTEIN 1 IN EARLY NEUTROPHIL RECRUITMENT AFTER SILICA EXPOSURE IN MICE

by

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Submitted to the Graduate Faculty of

the Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2014

UNIVERSITY OF PITTSBURGH

Graduate School of Public Health

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ABSTRACT

Occupational exposure to silica remains a major public health problem in both the US and other industrialized nations. We previously showed that silica-exposed female mice recruit greater numbers of inflammatory cells, express less secreted phosphoprotein 1 (SPP1) and are protected against the development of silica-induced pulmonary fibrosis (PF) compared to males at 14 days post-treatment. We therefore hypothesize that estrogen supplementation will protect male mice against silica-induced PF. Inflammation is implicated in the early pathogenesis of silicosis and animal models of silica-induced pulmonary fibrosis. In addition, intercellular adhesion molecule 1(ICAM-1), an important receptor in neutrophil recruitment, is induced after 24 h, and neutrophils are the predominant inflammatory cell type recruited into the lungs after 3 days of silica exposure in mice, before any overt fibrosis is observed. The roles of gender and SPP1 in silica-induced neutrophil recruitment at pre-fibrotic time points are unknown. We further hypothesize that female mice recruit greater numbers of neutrophils at pre-fibrotic time points, and that SPP1 regulation of ICAM-1 mediates gender-specific differences in neutrophil recruitment to the lungs following exposure to crystalline silica. We show here that estrogen-treated male mice recruit greater numbers of inflammatory cells, which are predominantly macrophages, express lower levels of SPP1, and are partially protected against the development of silica-induced PF. We also show that silica-exposed female mice recruit greater numbers of neutrophils, have more extensive tissue injury and

express less SPP1 and more ICAM-1 mRNA compared to exposed males at 3 days post-treatment. Interestingly, SPP1 deficiency does not influence neutrophil recruitment and lung tissue injury and upregulates ICAM-1 mRNA expression at 3 days post-silica exposure. We therefore suggest that the estrogen-mediated regulation of SPP1 may play a role in the gender-specific differences in silica-induced PF in mice. In addition, SPP1-mediated regulation of ICAM-1 does not account for the gender-specific differences in neutrophil recruitment into the lungs at pre-fibrotic time points. This study broadens the knowledge of silicosis, may provide insight into possible therapeutic measures to slow the progression of or cure silicosis and contributes to public health strategies and reduces the global burden of silicosis.

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PREFACE

I would like to express my deep gratitude to my academic advisor Dr. Cheryl Fattman for her guidance, encouragement and useful critique. I would not have completed this research without her support.

I would also like to express my appreciation to members of my dissertation committee: Dr. Bruce Pitt, committee chair; Dr. George Leikauf, Dr. Aaron Barchowsky and Dr. Tim Oury for their valuable and constructive suggestions during the planning and development of this work.

I also wish to thank members of Dr. Fattman's laboratory, Joseph Latoche and Fabrizio Fazzi, for their technical support especially in the early stages of my research. Thanks to Dr. Kiflai Bein and Vince Concel for helping me especially with RT-PCR.

Thanks to my mother Mrs. Hope Ufelle for her encouragement and Belchi Chiezey for being a true friend. I wish to thank my wife Tina Ufelle for her love, care and support throughout the course of the PhD program. I also wish to thank my pastors for their prayers. Finally, I wish to dedicate this dissertation to my late father Mr. Nehemiah Ufelle who taught me to work hard and endure to the finish line. To God be the glory.

1.0 INTRODUCTION

1.1 SILICOSIS AND PUBLIC HEALTH

Silicosis is a form of pulmonary fibrosis that results from the inhalation of free silica usually in occupational settings (Cassel et al., 2008; Greenberg, Waksman, & Curtis, 2007; Joshi & Knecht, 2013; Pryhuber, Huyck, Baggs, Oberdorster, & Finkelstein, 2003; Thomas & Kelley, 2010). Despite occupational regulations, silica exposure is still prevalent and an estimated 200-300 persons per year die from silicosis in the US (Greenberg et al., 2007), while the mortality from silicosis in China is much higher (Ramsgaard et al., 2010). Although mortality from silicosis declined between 1968 and 2007 (Figure 1) (CDC, March 2012), more than 2 million employees are still exposed to silica according to the Occupational Safety and Health Administration (OSHA) (Thomas & Kelley, 2010). Moreover, the number of deaths associated with silicosis is believed to be underestimated, as reflected in the annual report on silicosis (Thomas & Kelley, 2010). While age-adjusted mortality rates in the USA decreased from 8.9 per million in 1968 to 0.7 per million in 2004, silicosis deaths in young adults (15 – 44 years) have not declined due to recent intense exposures among the coal mining community (Leung, Yu, & Chen, 2012).

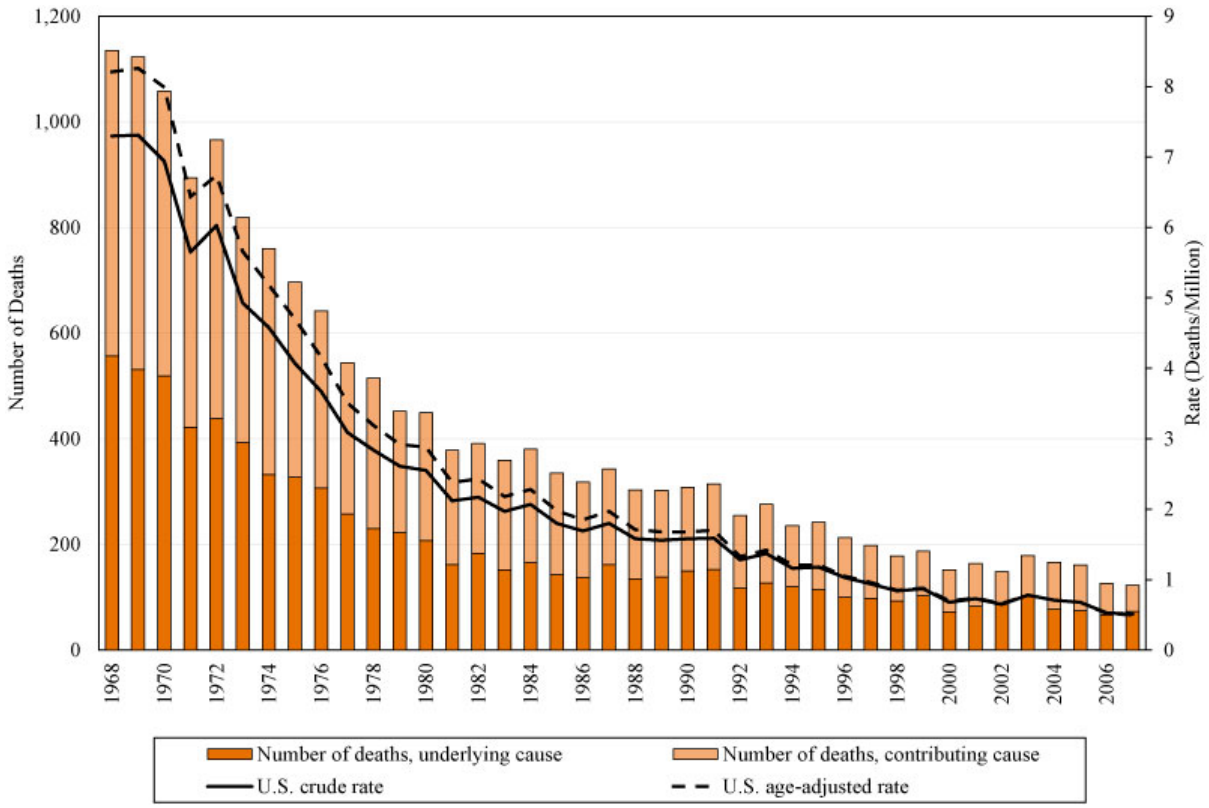
Occupational exposure to silica is the primary cause of silicosis, and Table 1 lists the jobs that are a potential source of silica exposure (OSHA, 2013; Thomas & Kelley, 2010). The consequence of silicosis traverses beyond the US and affects other developing countries, but efforts to control silica exposure has been frustrated by industry opposition (Thomas & Kelley, 2010). Silica caused the worst industrial disaster in the US at Hawk's Nest, West Virginia between 1930-1931 (Thomas & Kelley, 2010). In Europe,

more that 3 million workers are exposed to silica. However, it is worse in recently industrialized countries like China and India, where more than 23 million and 10 million workers are exposed to silica, respectively (W. Chen et al., 2012). Furthermore, it has been shown that exposure to silica is associated with increased mortality, not only from respiratory causes but cardiovascular end points as well (W. Chen et al., 2012). An increased incidence of silicosis has been reported in Spain as result of housing boom due to the use of new construction material quartz conglomerate (Perez-Alonso et al., 2014). Finally, individuals exposed to silica are predisposed to developing tuberculosis and subsequently lung cancer, thereby worsening a major public health concern (Castranova & Vallyathan, 2000; Kulkarni, 2007; Leung et al., 2012; OSHA, 2013). Although, silicosis currently cannot be cured, it is still preventable (Thomas & Kelley, 2010). The current exposure guidelines, which have not been updated for 40 years, state that the permissible exposure level is 100 µg of crystalline silica per cubic meter of air, averaged over 8 h (Cressey, 2014; OSHA, 2013; Thomas & Kelley, 2010). However, recent data suggest that this exposure level does not sufficiently protect workers and OSHA has recommended that current standard be reduced to 50 $\mu g/m^3$ (Cressey, 2014; OSHA, 2013). Silicosis is preventable and several measures have been recommended to ensure minimal occupational exposure including: hazard recognition, air sampling, engineering control, regular medical examination, education, and respiratory protection (personal protective devices) (Hamel, 2013; OSHA, 2013). These recommendations will promote public health and minimize the duration and amount of exposure and reduce mortality associated with silicosis (OSHA, 2013).

Table 1: The list of jobs that are potential sources of silica exposure.

Job/industry	Description
Construction	Sand blasting, rock drilling, masonry work, jack hammering, tunneling
Mining	Cutting or drilling through sand stone and granite
Glass manufacturing	
Agriculture	
Ship yards	Abrasive blasting
Foundry	Grinding, molding, shakeout, core room
Ceramics, clay and pottery	
Stone cutting	Sawing, abrasive blasting, chipping, grinding
Rail road	Setting and laying tracks
Manufacturing	Use of abrasive, soap and detergent

(OSHA, 2013; Thomas & Kelley, 2010)



(CDC, March 2012).

Figure 1: Silicosis: Mortality 1968-2007 in the US.

1.2 PATHOGENESIS OF SILICOSIS

Inflammation plays a key role in the pathogenesis of silica-induced pulmonary fibrosis (Bissonnette & Rola-Pleszczynski, 1989). Upon inhalation, silica particles are phagocytized by neutrophils and macrophages recruited to the lungs. It is believed that efficient scavenging of silica particles by macrophages is key to limiting overall lung injury and fibrosis (Gilberti, Joshi, & Knecht, 2008; Greenberg et al., 2007; Hamilton, Thakur, & Holian, 2008), and one of the driving forces behind the silica-induced lung injury may be the decreased phagocytic capacity of silica-laden neutrophils and macrophages (Adamson & Bowden, 1984). The inhalation of silica is associated with the generation of

reactive oxygen species (ROS), which play key roles in the pathogenesis of silicosis (Castranova & Vallyathan, 2000; Fazzi et al., 2014). The ROS produced by silica exposure can directly affect lung cells leading to peroxidation of lipids in and damage to cell membranes (Castranova & Vallyathan, 2000). Silica-laden macrophages induce an inflammatory response by activating the NALP3 inflammasome to produce IL-1β (Cassel et al., 2008; Joshi & Knecht, 2013; Ramsgaard et al., 2010). In addition, silica exposure has been associated with the production of pro-fibrotic cytokines such as IL-6 (Vanhee, Gosset, Boitelle, Wallaert, & Tonnel, 1995) and TGFβ (Rabolli et al., 2011; Ramsgaard et al., 2010).

IL-1β and TNFα play an important role in the pathogenesis of silica-induced pulmonary fibrosis in mice (Davis, Pfeiffer, & Hemenway, 1998) and humans (Y. W. Wang, Lan, Yang, Wang De, & Kuang, 2012). Previous studies have shown that neutralization of IL-1β inhibits TGFβ-1, collagen 1 and fibronectin gene expression, and subsequently attenuates silica-induced pulmonary fibrosis (Guo et al., 2013). In addition, inhibition of the Th2 immune response, via neutralization of IL-17A, decreases IL-1β and IL-6 secretion and may influence silica-induced inflammation and fibrosis in mice (Y. Chen et al., 2014). IL-17A is a pro-fibrotic cytokine that induces the secretion of collagen in a TGFβ-1-dependent manner and when neutralized, attenuates bleomycin-induced pulmonary fibrosis (Mi et al., 2011). TGFβ-1 and TNFα are important mediators of silicosis development and are elevated in the peripheral blood of silicosis patients (Miao et al., 2011). Furthermore, there is a positive correlation between Smad2/3 expression, which are transcription factors activated by TGFβ-1, and the hydroxyproline content of the lungs (as a measure of fibrosis) in response to silica (Ji, Yang, Wang, & Ding, 2004). Finally,

IL-10 exerts pro-fibrotic activities through upregulation of the Th2 response and induction of the pro-fibrotic cytokines IL-4 and IL-13 following silica exposure (Barbarin, Xing, Delos, Lison, & Huaux, 2005).

1.3 GENDER AND PULMONARY FIBROSIS

Idiopathic PF is more common in males (Brass et al., 2010; Carey, Card, Voltz, Arbes, et al., 2007; Iwai, Mori, Yamada, Yamaguchi, & Hosoda, 1994) and women are known to have better outcomes than men (Gribbin et al., 2006), suggesting that gender influences the severity and outcome of pulmonary fibrosis. For example, an earlier study showed differences in lung function between male and female mice in response to bleomycin that was worsened by androgen treatment (Voltz et al., 2008). Naive adult male C57BL/6 mice had 25% more lung hydroxyproline content compared to age matched females. This difference was not observed in androgen receptor-deficient mice, suggesting a possible role for the androgen receptor in collagen deposition during lung maturation (Carey, Card, et al., 2007b). In addition to the above observations, female mice treated with bleomycin had less severe pulmonary fibrosis compared to exposed males (Brass et al., 2010; Carey, Card, et al., 2007b; Redente et al., 2011b; Townsend, Miller, & Prakash, 2012). It was also observed that estradiol restored diminished bleomycin-induced pulmonary fibrosis in ovariectomized rats (Carey, Card, et al., 2007b). Finally, we previously showed that female mice had less pulmonary fibrosis and more inflammatory cells compared to male mice in response to silica, while ovariectomized mice showed fibrosis similar to males, further supporting a role for gender and estrogen in silica-induced pulmonary fibrosis (Brass et al., 2010).

1.4 LUNG NEUTROPHIL RECRUITMENT

Neutrophils are the first responders to infection and tissue injury (Jennings & Knaus, 2014) including silica exposure (Hornung et al., 2008). Upon inhalation, silica particles are phagocytized by recruited neutrophils and macrophages in the lungs.

At 14 days post-silica exposure, macrophages are the predominant inflammatory cell recruited into the lungs (Brass et al., 2010; Ramsgaard et al., 2010). However, at earlier time points, neutrophils are the predominant inflammatory cell (Nario & Hubbard, 1997; Sato, Shimosato, Alvord, & Klinman, 2008). Silica particles induce inflammatory responses by activating the NALP3 inflammasome (Cassel et al., 2008; Ramsgaard et al., 2010) to produce IL-1β. Interestingly, neutrophil influx is mediated by IL-1β, which is produced in higher amounts upon silica exposure in mice (Hornung et al., 2008). The recruitment of neutrophils to the site of injury involves multiple steps from chemotaxis to cellular adhesion and trans-endothelial migration (TEM) (Basit et al., 2006; Greenberger et al., 1996; Ochietti et al., 2002; L. Yang et al., 2005). Intercellular adhesion molecule 1 (ICAM-1) is known to be critical in neutrophil recruitment through its role in neutrophil adhesion and TEM to sites of injury (Basit et al., 2006; Ochietti et al., 2002; L. Yang et al., 2005). ICAM-1 is constitutively expressed on lung epithelial and endothelial cells and is upregulated by IL-1 and TNFα. Interestingly, TNFα is upregulated in the BAL of silicosis patients (Vanhee et al., 1995) and in silica-exposed mice (Ohtsuka et al., 1995). TEM of neutrophils is mediated through ICAM-1 on endothelial cells, but the retention of neutrophils at the epithelial surface is due to epithelial cell-expressed ICAM-1 (Nario & Hubbard, 1996). The CD11/CD18-dependent and -independent pathways are the two possible routes through which neutrophils adhere to pulmonary microvascular endothelial

cells and migrate to the distal spaces in the lungs (Doerschuk, Tasaka, & Wang, 2000; Long, 2011). IL-1 (Hornung et al., 2008) and TNFα (Vanhee et al., 1995) activate the CD11/CD18 dependent pathway, which leads to the production of ICAM-1 (Doerschuk et al., 2000; Long, 2011; Mizgerd, 2002). MIP-2 is a potent chemotactic factor for neutrophils (Driscoll, 1994) and it is one of several cytokines involved in mediating neutrophillic inflammation following a single instillation of crystalline silica (Yuen, Hartsky, Snajdr, & Warheit, 1996).

1.5 GENDER AND NEUTROPHIL RECRUITMENT

A gender bias in neutrophil recruitment has been observed. For example, female rats and mice recruit fewer neutrophil to sites of burn injury compared to males (M. D. Bird, Karavitis, & Kovacs, 2008). Contrary to this, women and female animals recruit greater numbers of neutrophils compared to males in alcoholic acute liver injury (Eagon, 2010) and neutrophil counts are reported to be higher in females of all ages in severe acute inflammatory conditions (Casimir, Lefevre, Corazza, & Duchateau, 2013). It is known that estrogen increases neutrophil survival (Molloy et al., 2003) and significantly increases lung inflammation and neutrophil recruitment in *Pseudomonas aeruginosa* pneumonia in a mouse model of cystic fibrosis (Y. Wang, Cela, Gagnon, & Sweezey, 2010), but suppresses neutrophil recruitment in LPS-induced acute lung injury in mice (Speyer et al., 2005). Taken together, this information shows the ambiguity of the role of gender on inflammation, and that the effects of estrogen on inflammation depend upon both the time point analyzed and the mode of injury.

1.6 SECRETED PHOSPHOPROTEIN 1 (SPP1)

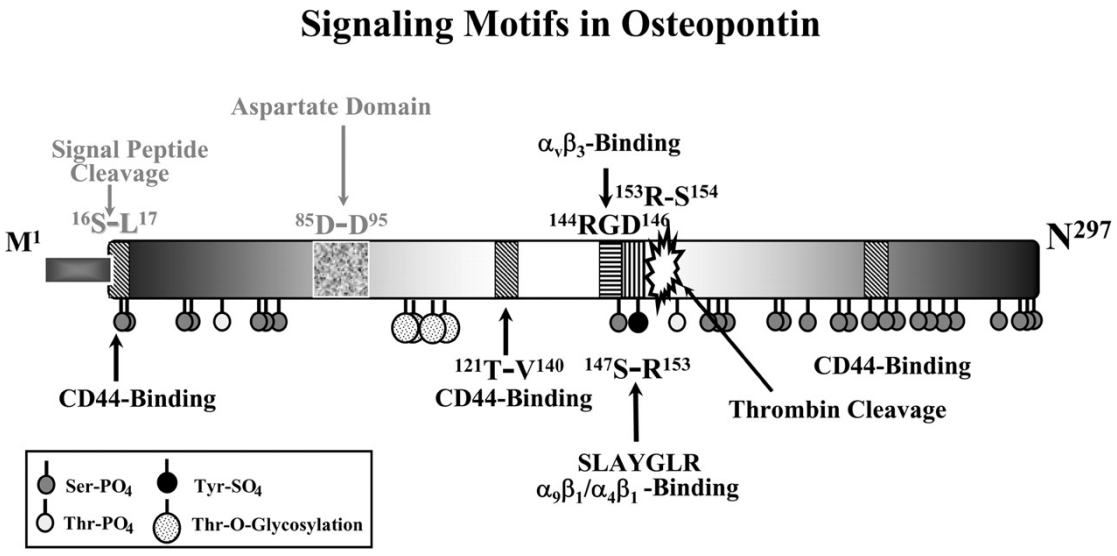
Osteopontin or SPP1 is a highly o-glycosylated phosphoprotein that is expressed in a number of cells and tissues and is secreted into many body fluids (Buback, Renkl, Schulz, & Weiss, 2009; Serlin et al., 2006; Sodek, Ganss, & McKee, 2000; K. X. Wang & Denhardt, 2008; Zhang, Pan, Huang, Weber, & Zhang, 2011). It is an aspartate-rich protein, which contains several protein interaction binding domains: an RGD (arginine-glycine-aspartate) integrin-binding domain, a heparin-binding motif, cleavage sites for thrombin and matrix metalloproteinases, and a CD44 binding site at the C-terminus of the protein (Buback et al., 2009; Sodek et al., 2000; K. X. Wang & Denhardt, 2008; Zhang et al., 2011). SPP1 is expressed as a 33 kDa nascent protein, but post-translational modifications increase the molecular weight to approximately 44 kDa (Sodek et al., 2000; K. X. Wang & Denhardt, 2008). The full-length mouse SPP1 consists of 297 amino acids, while the full-length human form has 314. Both human and mouse SPP1 exist as a number of cleavage products and splice variants (Inoue & Shinohara, 2011; Sodek et al., 2000; K. X. Wang & Denhardt, 2008). SPP1 binds to integrins via the RGD (for integrins ITGAV-B3, ITGAV-B5, ITGAV-B1, ITGAV-B6, ITGAV-B1, ITGA5-B1, ITGA8-B1) and SVVYGLR (SLAYGLR in mice) (for integrins ITGA9-B1, and ITGA4-B1) motifs, respectively. In addition, SPP1 also interacts with integrin ITGAX-B2 and CD44, especially the v6 and v7 variants (Denhardt, Giachelli, & Rittling, 2001; Inoue & Shinohara, 2011; O'Regan, 2003; Wai & Kuo, 2004).

SPP1 undergoes numerous post-translational modifications that define its biological activities such as phosphorylation, glycosylation, polymerization, transglutaminase 2-mediated alterations and proteolytic cleavage by proteases such as

thrombin and cathepsin D (Arjomandi et al., 2011; Mazzali et al., 2002). Figure 2 shows signaling motifs in SPP1 (Sodek, Batista Da Silva, & Zohar, 2006) SPP1 plays a role in many biological and pathological processes including but not limited to: development, wound healing, immunological responses, tumorigenesis, bone resorption and calcification (Sodek et al., 2000). SPP1 mediates cell adhesion, migration, tumor invasion, has T- helper 1 (Th1) cytokine function, and anti-apoptotic effects via interaction with the integrins and CD44 (Buback et al., 2009; Denhardt, Noda, O'Regan, Pavlin, & Berman, 2001). In the normal lung, SPP1 is expressed by bronchial epithelial cells and scattered alveolar macrophages, but is highly expressed by injured epithelial cells, alveolar and interstitial macrophages, T cells and pulmonary vascular endothelium in pathological conditions. In macrophages, SPP1 is thought to play a role in phagocytosis because of its high expression in actively phagocytizing macrophages (Denhardt, Giachelli, et al., 2001). Furthermore, SPP1 is positively associated with macrophage recruitment, as SPP1-deficient macrophages fail to migrate to chemoattractants such as formyl-met-leu-phe (O'Regan, 2003). SPP1 exhibits pro-inflammatory and anti-inflammatory effects (Lund, Giachelli, & Scatena, 2009) and can have opposing effects, depending upon the cellular context (L. Bird, 2007; Xanthou et al., 2007). SPP1 is chemotactic to neutrophils (van der Windt, Hoogerwerf, de Vos, Florquin, & van der Poll, 2010) but loss of SPP1 does not affect the phagocytic ability or the generation of ROS by neutrophils (Koh et al., 2007). In addition, SPP1 deficiency does not influence neutrophil recruitment in response to Streptococcal pneumonia (van der Windt et al., 2011). Many studies suggest that integrin alpha 9-beta 1(ITGA9-B1) binding to the SLAYGLR sequence of SPP1 mediates neutrophil recruitment (Banerjee, Lee, & Ramaiah, 2008; Taooka, Chen, Yednock, &

Sheppard, 1999; Uede, 2011; Yokosaki et al., 1999). Additionally, SPP1 induces the switching of normal lung fibroblasts to the pro-fibrogenic myofibroblast phenotype in response to allergens (Kohan, Breuer, & Berkman, 2009a). Human fibroblasts have not been shown to express SPP1, though rat cardiac fibroblasts highly express SPP1 (O'Regan, 2003).

SPP1 expression is affected by a number of substances including hormones, cytokines and growth factors. Transcription of the SPP1 gene is regulated by transactivation of cis-acting elements in the gene promoter (El-Tanani et al., 2006). Interestingly, estrogen inhibits SPP1 production in vascular smooth muscle cells (Li, Chen, Kelpke, Oparil, & Thompson, 2000). Also, in a rodent model of alcoholic steatohepatitis, low doses of estrogen downregulate the expression of SPP1, while high doses of estrogen upregulates SPP1expression (Banerjee, Rose, Johnson, Burghardt, & Ramaiah, 2009).



(Sodek et al., 2006)

Figure 2: Signaling Motifs in SPP1.

1.7 SPP1 AND PULMONARY FIBROSIS

Previous studies indicate that upregulation of SPP1 in wound inflammatory responses hinders tissue repair and contributes to wound scarring (fibrosis) (Mori, Shaw, & Martin, 2008). SPP1 expression is also upregulated in bronchoepithelial cells after exposure to chrysolite asbestos and decreased asbestos-induced injury and inflammation was observed in SPP1-deficient mice (Sabo-Attwood et al., 2011). The development of fibrosis is associated with increased expression of SPP1 mRNA and protein following intratracheal instillation of bleomycin to mice. In addition, SPP1 is strongly expressed in the alveolar macrophages accumulating in the lungs. Recombinant SPP1 enhanced migration, adhesion, and PDGF-mediated DNA synthesis in a murine fibroblast NIH 3T3 cell line (Takahashi et al., 2001a) and also significantly increased the migration and proliferation of both primary human lung fibroblasts and A549 alveolar epithelial-like cells (Pardo et al., 2005). Analyses using oligonucleotide arrays showed that SPP1 is elevated in the BAL and lungs of IPF patients relative to controls (Pardo et al., 2005). Further analysis suggests a significant interaction between SPP1 and MMP-7 expression [32]. Finally, we have shown that SPP1 mRNA and protein are elevated in both the BAL and lung tissue of mice treated with silica and that male mice have higher BAL and tissue-associated SPP1 protein and mRNA expression compared to female mice at 14 days post-treatment (unpublished data).

1.8 SUMMARY

Despite regulations, an estimated 200-300 persons die of silicosis per year in the US. There is no effective treatment for silicosis, leaving prevention as the only option presently available. A gender bias exists in the severity of silica-induced pulmonary

fibrosis and inflammation. Estrogen and gender play a role in neutrophil recruitment, inflammation and pulmonary fibrosis. In addition, SPP1 plays a role in neutrophil recruitment and modulates the severity of IPF and fibrosis in animal models. Furthermore, ICAM1 is known to play a key role in the recruitment of neutrophils into the lungs following exposure to crystalline silica. Taken together, understanding the role of estrogen in silica-induced PF and the roles of gender and SPP1 in silica-induced early neutrophil recruitment will yield insights into the progression and prevention of silicosis and, by extension, other forms of pulmonary fibrosis.

2.0 ESTROGEN SUPPLEMENTATION PARTIALLY PROTECTS MALE MICE AGAINST SILICA-INDUCED PULMONARY FIBROSIS

This is part of Latoche et al. Secreted phosphoprotein 1 contributes to the gender-specific sensitivity of mice to silica-induced pulmonary fibrosis, Environmental Health Perspective (under review).

2.1 ABSTRACT

2.1.1 Background

Gender is known to play a role in silica-induced pulmonary fibrosis. We previously showed that silica-exposed female mice have less severe pulmonary fibrosis compared to exposed males at 14 days post-treatment. We also showed that the extent of lung fibrosis in ovariectomized mice is similar to that of males at 14 days post-silica exposure. In addition, silica-exposed female mice express less SPP1 compared to exposed males at 14 days post-treatment. We hypothesize that estrogen supplementation will protect male mice against silica-induced pulmonary fibrosis.

2.1.2 Results

We pre-treated 8-10 weeks old male C57BL/6J mice with 17-β estradiol or olive oil as a vehicle control for 3 weeks. We then exposed the pretreated mice to 0.2g/ Kg body weight of crystalline silica by intratracheal instillation and assessed lung injury at 14 days post-treatment. Estrogen-treated male mice recruited a greater number of inflammatory cells, which were predominantly macrophages, and developed less extensive pulmonary fibrosis compared to vehicle-treated male mice at 14 days post-silica exposure. Furthermore, SPP1 expression was decreased in estrogen-treated male mice compared to vehicle-treated male mice at 14 days post-silica exposure.

2.1.3 Conclusion

We conclude that the estrogen-mediated regulation of SPP1 may play a role in the gender-specific differences in silica-induced pulmonary fibrosis in mice.

2.2 BACKGROUND

Silicosis is a form of pulmonary fibrosis (PF), resulting from the inhalation of silica dust during occupational exposures (Cassel et al., 2008; Greenberg et al., 2007; Joshi & Knecht, 2013; Pryhuber et al., 2003). Despite occupational regulations, silica exposure is still prevalent and an estimated 200 to 300 persons per year die from silicosis in the U.S (Greenberg et al., 2007), while the death rate in other countries such as China is much higher (Ramsgaard et al., 2010). Upon inhalation silica particles are phagocytized by alveolar macrophages and induce an inflammatory response through the activation of the NALP3 inflammasome (Cassel et al., 2008; Ramsgaard et al., 2010). It is believed that efficient scavenging of silica particles by macrophages is key to limiting the overall lung injury and fibrosis (Greenberg et al., 2007; Hamilton et al., 2008; Iwai et al., 1994). Silica exposure also induces the production of pro-fibrotic cytokines such as IL-6 (Vanhee et al., 1995) and TGFβ (Barbarin, Nihoul, et al., 2005; Gribbin et al., 2006).

A gender bias exists in pulmonary fibrosis. Idiopathic pulmonary fibrosis (IPF) is more common in men (Brass et al., 2010; Carey, Card, Voltz, Arbes, et al., 2007; Iwai et al., 1994) and women have better outcomes (Gribbin et al., 2006). In addition to this observation, bleomycin-induced lung fibrosis is less severe in female mice compared to males (Carey, Card, et al., 2007b; Redente et al., 2011b; Townsend et al., 2012). It was further observed that estradiol treatment diminishes bleomycin-induced pulmonary fibrosis in ovariectomized rats (Carey, Card, et al., 2007b). We also showed that female

mice develop less pulmonary fibrosis and recruit a greater number of inflammatory cells to the lungs in response to crystalline silica compared to males, while ovariectomized mice developed fibrosis to a similar extent as males (Brass et al., 2010), suggesting that female gender and by extension estrogen may play key roles in silica-induced pulmonary fibrosis.

An earlier study indicated that SPP1 is upregulated in wound inflammatory responses, hinders tissue repair and contributes to wound scarring/fibrosis (Mori et al., 2008). In addition, the progression of fibrosis was associated with an increase in SPP1 mRNA and protein expression following intratracheal instillation of bleomycin in mice, with SPP1 being strongly expressed in alveolar macrophages (Takahashi et al., 2001b). Furthermore, SPP1 is elevated in the lungs of IPF patients relative to normal lungs and increased in the bronchoalveolar lavage fluid (BALF) of IPF patients (Pardo et al., 2005). SPP1 is important in asbestos-induced pulmonary fibrosis in animal models, as many genes associated with pulmonary fibrosis are not upregulated in SPP1-null mice (Sabo-Attwood et al., 2011). Our laboratory determined that expression of SPP1 is higher in the BALF and tissue of silica-exposed male mice compared to exposed females at 14 days post-treatment. In addition, SPP1-null mice develop less silica-induced pulmonary fibrosis at the same time point (unpublished data). Based on the existing data, we hypothesize that SPP1 is an estrogen-regulated pro-fibrotic mediator of silica-induced pulmonary fibrosis.

The role of estrogen in silica-induced pulmonary fibrosis is currently unclear. We therefore wished to determine if estrogen supplementation of male mice protects against silica-induced pulmonary fibrosis through downregulation of SPP1. We show here that

estrogen supplementation of male mice protects against silica-induced SPP1 increases and pulmonary fibrosis at 14 days post-treatment.

2.3 RESULTS

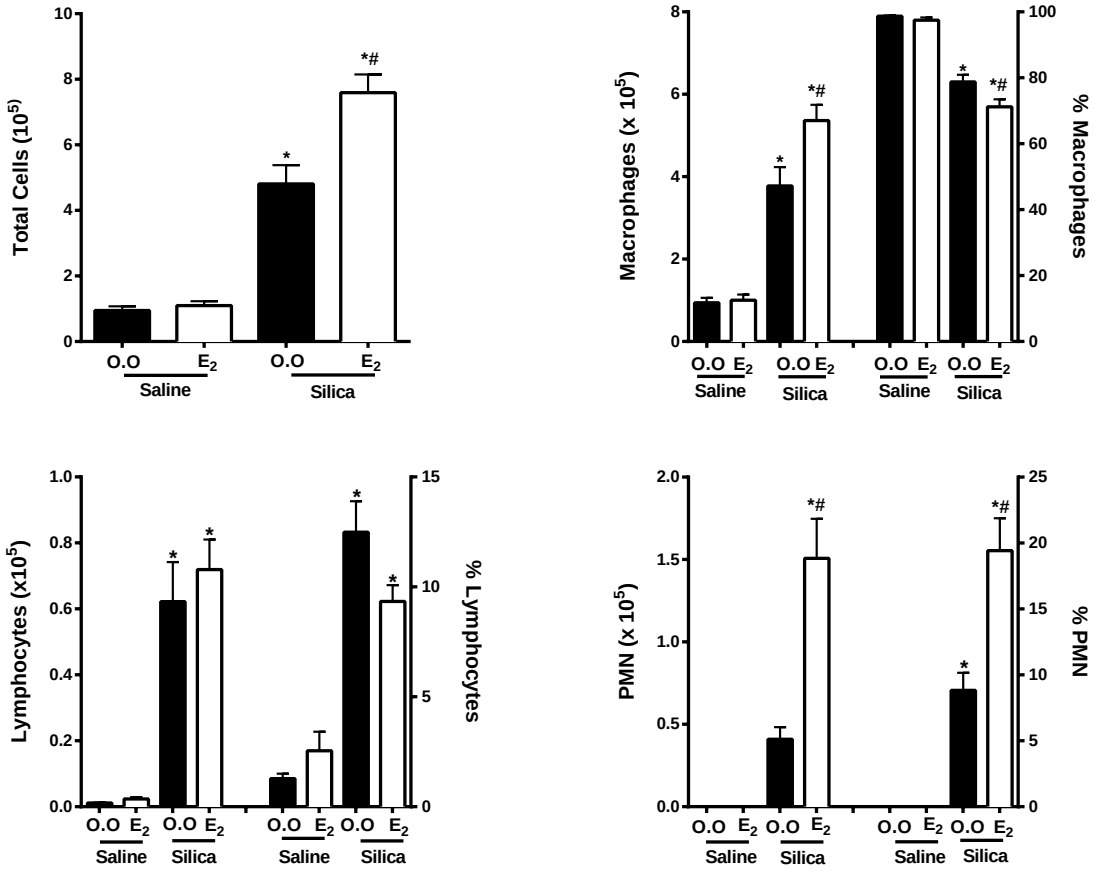
Estrogen-treated male mice have a greater inflammatory response to silica compared to olive oil-treated mice at 14 days post-silica exposure.

We previously showed that silica-exposed female mice recruit greater numbers of inflammatory cells predominantly macrophages, to the lung compared to silica-exposed male mice at 14 days post-treatment. We also demonstrated that the inflammatory response of ovariectomized mice was similar to male mice (Brass et al., 2010).

To determine the direct effect of estrogen on the silica-induced inflammatory response, we pretreated 8-10 weeks old C57Bl/6J male mice with 250 ng of 17β-estradiol or the equivalent volume of the olive oil vehicle for 21 days, followed by intratracheal administration of 0.2g/Kg body weight of freshly fractured crystalline silica.

We assessed inflammation at 14 days post-silica exposure in BALF by differential analysis of Protocol Hema 3-stained inflammatory cells. The BALF analysis (Fig: 3A) shows that silica-treated mice recruit greater total numbers of inflammatory cells compared to saline-exposed control mice in both the estrogen-treated and olive oil-treated groups. In addition, estrogen-treated silica-exposed mice recruit greater total numbers of inflammatory cells compared to the olive oil-treated silica-exposed mice (Fig: 3A). Furthermore, silica-exposed mice recruit greater numbers of macrophages compared to saline-exposed mice in both the estrogen-treated and olive oil-treated groups (Fig: 3B). Interestingly, estrogen-treated silica-exposed mice recruit greater numbers of macrophages compared to olive oil-treated silica-exposed mice (Fig: 3B). In

addition, silica-treated mice recruit greater total numbers of lymphocytes compared to the respective saline-exposed control mice in both the estrogen-treated and olive oil-treated groups (Fig: 3C). However, lymphocyte recruitment is not significantly different between estrogen-treated and olive oil-treated silica-exposed mice (Fig: 3C). Finally, silica-treated mice recruit greater total numbers of neutrophils compared to saline-exposed mice in both the estrogen-treated and olive oil-treated groups (Fig: 3D). Interestingly, estrogen-treated silica-exposed mice recruit greater numbers of neutrophils compared to olive oil-treated silica-exposed mice at 14 days post exposure (Fig: 3D).

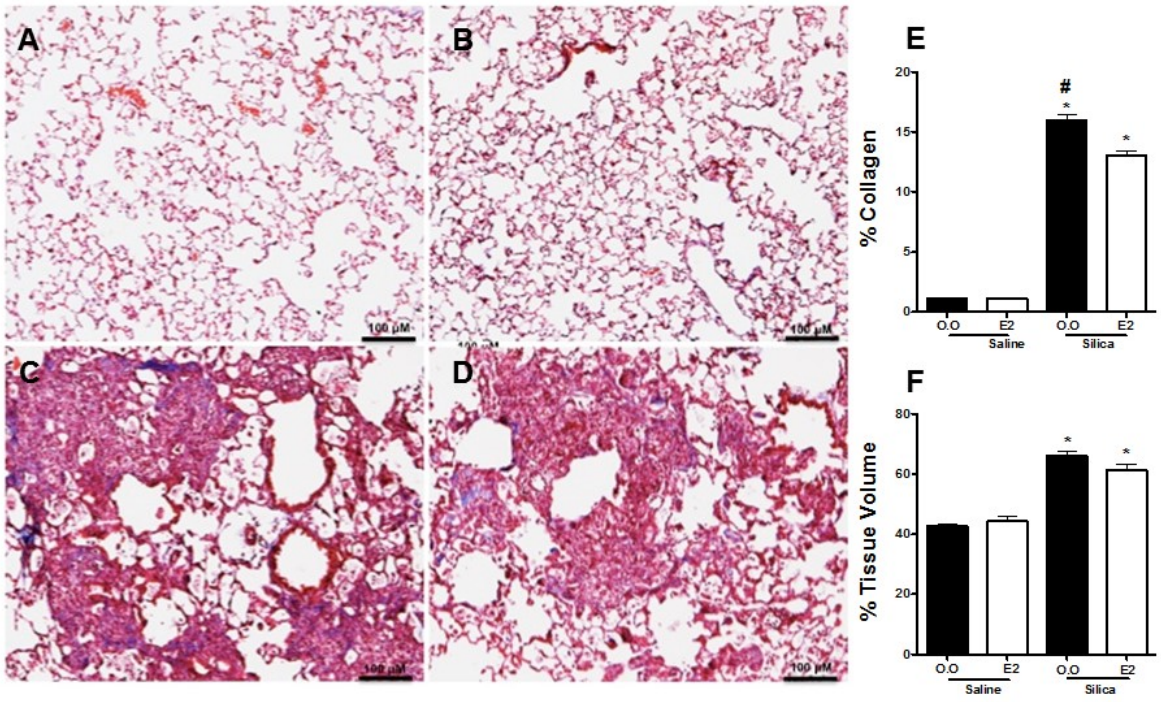


This panel shows the total number of inflammatory cells in whole lung lavage at 14 days post-silica exposure (A). The number and percentage of total inflammatory cells that are macrophages (B), lymphocytes (C), and neutrophils (D) in the BALF of estrogen (E2)- and vehicle (O.O)-treated male mice at 14 days post-silica treatment are shown. N ≥ 5 per group, * indicates $p<0.05$ vs. same-treatment saline, # indicates $p<0.05$ vs. olive oil-treated silica-exposed mice by one-way ANOVA followed by Bonferroni correction for multiple comparisons.

Figure 3: Estrogen-treated male mice have a greater inflammatory response to silica compared to controls at 14 days post-exposure.

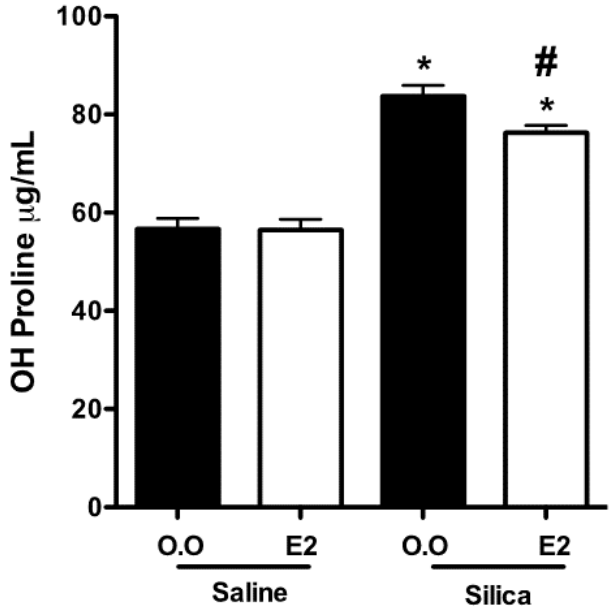
Silica-induced fibrosis is reduced in estrogen-treated mice compared to olive oil-treated mice at 14 days post-exposure.

Our earlier studies showed that silica-exposed female mice are partially protected from pulmonary fibrosis compared to exposed males and that ovariectomized mice develop fibrosis to a similar extent as males (Brass et al., 2010). We assessed lung collagen deposition by Mason's trichrome staining and hydroxyproline analysis (Figure 4). We show that silica-exposed mice have increased collagen deposition and fibrosis as assessed both histologically and by hydroxyproline analysis compared to the respective control for both estrogen-treated and olive oil-treated mice (Fig: 4 C vs. A; D vs. B; E, F, and Fig: 5). Interestingly, estrogen-treated silica-exposed mice show reduced collagen deposition and fibrosis compared to olive oil-treated silica-exposed mice at 14 days post-exposure (Fig: 4 D vs. C; E and Fig: 5). NB: the blue representing collagen is clearer on the microscope than it is on file.



This panel shows Mason' trichrome stained lung tissue from (A) olive oil-treated saline-exposed mice, (B) estrogen-treated saline-exposed mice, (C) olive oil-treated silica-exposed mice, and (D) estrogen-treated silica-exposed mice at 14 days post-exposure. The percentage of collagen in lung tissue at 14 days post-silica treatment is shown in (E), the percentage of involved tissue (tissue volume) at 14 days post-silica treatment is shown in (F). N ≥ 5 per group, * indicates $p<0.05$ vs. same-treatment saline, # indicates $p<0.05$ vs. olive oil-treated silica-exposed mice by one-way ANOVA followed by Bonferroni correction for multiple comparisons. NB: E2 = estrogen, O.O = olive oil.

Figure 4: Silica-induced pulmonary fibrosis is reduced in estrogen-treated male mice at 14 days post-exposure.



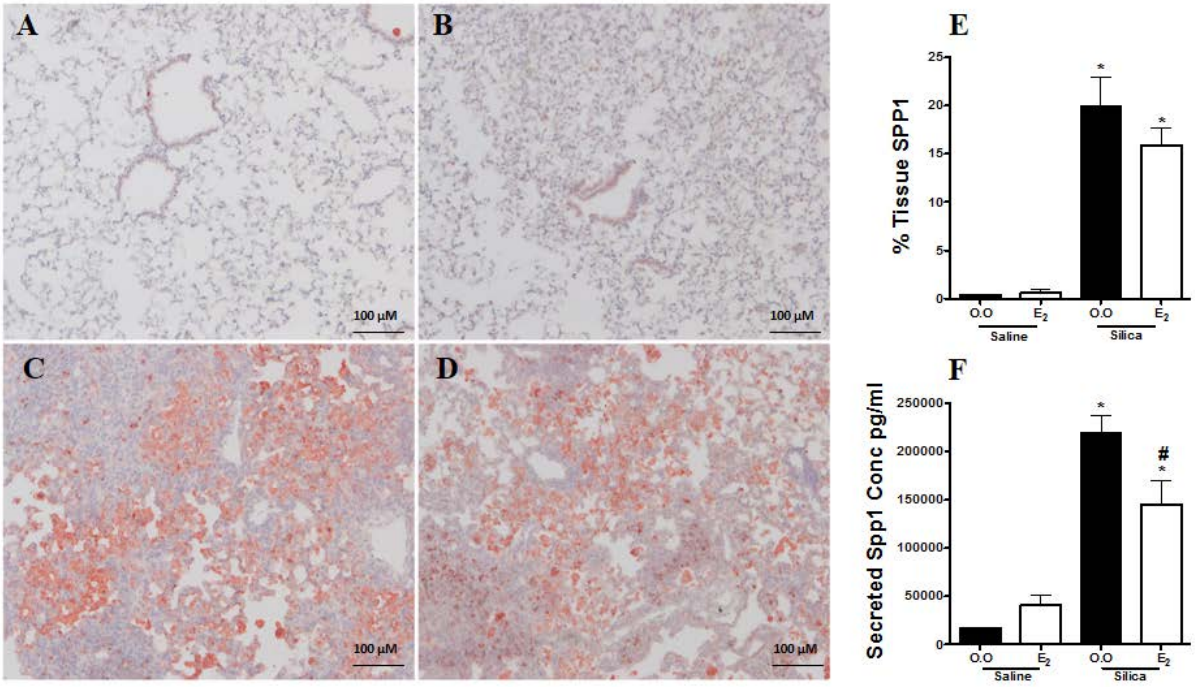
Hydroxyproline levels, as a measure of collagen deposition, are shown. N ≥ 5 per group. * indicates $p<0.05$ vs. same-treatment saline, # indicates $p<0.05$ vs. olive oil-treated silica-exposed mice by one-way ANOVA followed by Bonferroni correction for multiple comparisons. NB: E2 = estrogen, O.O = olive oil.

Figure 5: Lung hydroxyproline levels are reduced in estrogen-treated compared to olive oil-treated male mice at 14 days post-silica exposure.

Estrogen regulates SPP1 protein expression in male mice.

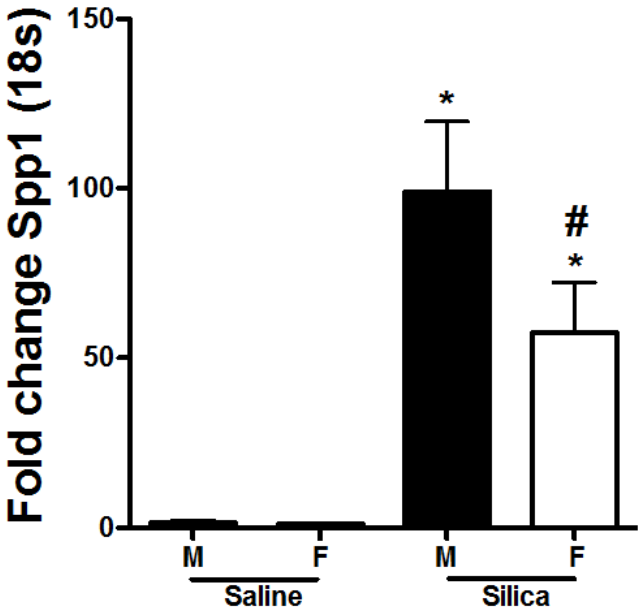
Previous work showed that estrogen regulates SPP1expression (Li et al., 2000; Miyajima, Hayashi, Saito, Iida, & Matsuoka, 2010). In addition, results from our laboratory show that silica-exposed female mice express less SPP1 compared to exposed males at 14 days post-treatment (unpublished data).

Figure 6 shows that silica-exposed mice express higher levels of secreted SPP1 compared to saline-exposed controls for both estrogen-treated and olive oil-treated mice respectively (Fig: 6 C vs. A; D vs. B; E, and F). Furthermore, SPP1 expression is decreased in estrogen-treated silica-exposed mice compared to olive oil-treated silica-exposed mice at 14 days post-treatment (Fig: 6 F). However, the amount of tissue-associated SPP1 is not significantly different between estrogen-treated and olive oil-treated silica-exposed mice (Fig: 6 D vs. C; and E). In support of the above data, SPP1 mRNA levels are reduced in silica-exposed female mice compared to exposed males at 14 days post-treatment (Fig: 7).



This panel shows immunohistological staining for SPP1 in lung tissue from (A) olive oil-treated saline-exposed mice, (B) estrogen-treated saline-exposed mice, (C) olive oil-treated silica-exposed mice, and (D) estrogen-treated silica-exposed mice at 14 days post-exposure. The percentage of tissue staining for SPP1 is shown in (E), and the level of secreted SPP1 in BALF is shown in (F). N ≥ 5 per group, * indicates $p<0.05$ vs. same-treatment saline, # indicates $p<0.05$ vs. olive oil-treated silica-exposed mice by one-way ANOVA followed by Bonferroni correction for multiple comparisons. NB: E2 = estrogen, O.O = olive oil.

Figure 6: Estrogen-treated silica-exposed male mice secreted less SPP1 compared to olive oil-treated silica-exposed mice.



SPP1 mRNA levels are shown. N ≥ 3 per group. * indicates $p<0.05$ vs. same-gender saline, # indicates $p<0.05$ vs. silica-exposed male mice, by one-way ANOVA followed by Bonferroni correction for multiple comparisons.

Figure 7: SPP1 mRNA expression is reduced in the lungs of silica-exposed female mice compared to male mice at 14 days post-treatment.

2.4 DISCUSSION

In this study, we determined the effect of estrogen on silica-induced pulmonary fibrosis in male mice. We demonstrate that estrogen-treated silica-exposed male mice recruit greater numbers of inflammatory cells, which were predominantly macrophages, compared to olive oil-treated silica-exposed mice at 14 days post-exposure. In addition, collagen deposition and fibrosis is reduced in estrogen-treated mice compared to olive oil-treated controls at 14 days post-silica exposure. Finally, we show reduced SPP1 expression in estrogen-treated silica-exposed male mice compared to olive oil-treated silica-exposed mice at 14 days after exposure. Therefore, we conclude that estrogen supplementation downregulates SPP1 expression and protects against silica-induced pulmonary fibrosis in male mice at 14 days post-treatment.

We previously showed that silica-exposed female mice recruit greater numbers of inflammatory cells which were also predominantly macrophages compared to exposed males at 14 days post-treatment (Brass et al., 2010). In addition, silica treatment of ovariectomized mice produced an inflammatory response similar to that of male mice (Brass et al., 2010). Consistent with the above findings, we show here that exposure of estrogen-treated male mice to silica produces a ‘female-like’ response: i.e. estrogen-treated males recruit greater numbers of inflammatory cells/macrophages compared to olive oil-treated males at 14 days post silica-exposure (Fig: 3 A/B). This result indicates that estrogen plays a role in inflammatory cell recruitment at 14 days post-silica exposure and suggests that estrogen increases macrophage recruitment to enhance the clearance of silica particle.

Other groups have reported a gender bias in pulmonary fibrosis. For example, idiopathic pulmonary fibrosis is more common in males (Iwai et al., 1994) and women have better outcomes than men (Gribbin et al., 2006), suggesting that gender plays a role in determining the incidence and progression of IPF. In addition, female mice treated with bleomycin develop less severe pulmonary fibrosis compared to treated males (Redente et al., 2011a). Furthermore, estradiol supplementation restored diminished bleomycin-induced pulmonary fibrosis in ovariectomized rats (Carey, Card, et al., 2007a). We previously showed that silica-induced pulmonary fibrosis was less severe in silica-exposed females compared to males, while the level of fibrosis in silica-treated ovariectomized mice was similar to males at 14 days post-exposure (Brass et al., 2010). Consistent with the above findings, we show that estrogen-treated male mice develop less severe silica-induced pulmonary fibrosis compared to olive oil-treated mice at 14 days post-treatment (Fig: 4 D vs. C; E, and Fig: 5). This indicates that estrogen plays a key role in silica-induced pulmonary fibrosis in mice.

Earlier studies have shown that SPP1 is important in IPF and animal models of pulmonary fibrosis. For example, SPP1 expression is upregulated after exposure to chrysolite asbestos and asbestos-induced injury and inflammation was decreased in SPP1-null mice (Sabo-Attwood et al., 2011). In addition, bleomycin-induced pulmonary fibrosis is associated with increased expression of SPP1 mRNA and protein (Takahashi et al., 2001b). Furthermore, SPP1 is elevated in the BALF and lungs of IPF patients relative to normal controls (Pardo et al., 2005). We previously showed that SPP1 levels are elevated in silica-exposed mice compared saline-treated control mice. We also showed that silica-exposed female mice express less SPP1 compared to exposed males,

and that silica-induced pulmonary fibrosis is reduced in SPP1 null mice (unpublished data). Estrogen differentially regulates SPP1 expression depending upon the cellular context. For example, estrogen inhibits SPP1 expression in rat vascular smooth muscle cells (Li et al., 2000). In addition, ovariectomy suppresses the expression of SPP1 in the rat kidney (Miyajima et al., 2010). Interestingly, we show here that SPP1 expression is reduced in the BALF of estrogen-treated male mice compared to olive oil-treated mice at 14 days post-silica exposure (Fig: 6F). There is also a reduction in tissue-associated SPP1 in estrogen-treated male mice compared to olive oil-treated mice at 14 days post-silica exposure, but this reduction was not statistically significant (Fig: 6 D vs. C, and E). Finally, silica-exposed female mice express less SPP1 mRNA compared to exposed males at 14 days post-treatment (Fig: 7). These results suggest that estrogen treatment suppresses SPP1 expression to protect against silica-induced pulmonary fibrosis in male mice at 14 days post-treatment.

A previous study showed that estradiol injection of ovariectomized rats restored diminished bleomycin-induced pulmonary fibrosis (Carey, Card, et al., 2007a). Future studies will assess the effect of estrogen in silica-induced pulmonary fibrosis in ovariectomized mice. Since ovariectomized mice develop silica-induced pulmonary fibrosis to a similar extent as males (Brass et al., 2010), we expect estradiol to reduce the sensitivity of ovariectomized mice to silica. We previously showed that estrogen receptor alpha (ERα) but not estrogen receptor beta (ERβ) is differentially expressed based on gender, with male mice having higher ERα levels compared to female mice at 14 days post-silica treatment (Brass et al., 2010). It has been established that a viral-vector-mediated siRNA can be delivered locally to silence ERα expression (Ribeiro et al., 2012)

and mice having a tissue-specific knockdown of ERα by Cre-LoxP technology are available. Therefore, we can further strengthen this study by assessing the effect of the conditional knock down of lung ERα on the development of silica-induced pulmonary fibrosis in mice.

In conclusion, we have shown that silica-exposed female mice develop less pulmonary fibrosis and have reduced lung SPP1 levels compared to exposed males at 14 days post-treatment. Consistent with these findings, we have shown that estrogen supplementation of male mice decreased silica-induced pulmonary fibrosis and lung SPP1 levels compared to vehicle-treated controls at 14 days post-treatment. Therefore, further exploration of estrogen-mediated silica-induced pulmonary fibrosis will provide more insight into the pathogenesis of silicosis.

3.0 THE ROLE OF GENDER AND SPP1 IN SILICA-INDUCED EARLY NEUTROPHIL RECRUITMENT IN MICE

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3.1 ABSTRACT

3.1.1 Background

Inflammation is implicated in the early pathogenesis of silicosis and animal models of silica-induced pulmonary fibrosis. Lung inflammation is often present prior to the development of fibrosis and therefore may initiate the fibrotic process. Intercellular adhesion molecule 1 (ICAM-1), an important receptor for neutrophil migration from the circulation into tissues, is induced after 24 h of silica exposure in mice. In addition, neutrophils are the predominant inflammatory cell type recruited into the lungs after 3 days of silica exposure. We have earlier demonstrated that silica-treated female mice recruit a greater numbers of inflammatory cells, produce lower levels of the cytokine secreted phosphoprotein 1 (SPP1), and develop less pulmonary fibrosis compared to male mice at 14 days post-exposure. The role of gender and SPP1 in neutrophil recruitment at pre-fibrotic time points is unknown. We therefore hypothesized that female mice recruit greater numbers of neutrophils at pre-fibrotic time points compared to males and that SPP1 regulation of ICAM-1 mediates, in part, the gender-specific differences in neutrophil recruitment to the lungs following exposure to crystalline silica.

3.1.2 Results

Silica-exposed female mice recruit greater numbers of neutrophils and have a greater extent of lung injury compared to exposed males at 3 days post-treatment. In addition, silica-exposed female mice express more ICAM-1 mRNA compared to exposed males at pre-fibrotic time points. Furthermore, silica-exposed female mice express lower levels of SPP1 compared to exposed males at pre-fibrotic time points. To determine if SPP1 plays a role in neutrophil recruitment at 3 days post-exposure, we treated C57BL/6L

wild type and SPP1 (-/-) mice with silica. SPP1 deficiency does not influence neutrophil recruitment and the extent of lung injury. Finally, SPP1 deficiency upregulates ICAM-1 mRNA expression at 3 days post-silica exposure.

3.1.3 Conclusion

SPP1 levels are higher in male mice, but the SPP1-mediated suppression of ICAM-1 does not support the recruitment of fewer neutrophils in males compared to females at 3 days post-silica exposure.

3.1.4 Keywords

silica, intercellular adhesion molecule 1, secreted phosphoprotein 1, gender, pre-fibrotic, lung

3.2 BACKGROUND

Silicosis is a form of pulmonary fibrosis (PF), which results from the inhalation of silica dust during occupational exposure (Cassel et al., 2008; Greenberg et al., 2007; Joshi & Knecht, 2013; Pryhuber et al., 2003). Despite occupational regulations, silica exposure is still prevalent and an estimated 200 to 300 persons per year die from silicosis in the U.S (Greenberg et al., 2007). Because of its occupational nature, silicosis occurs predominantly in males and sensitivity to other forms of PF has been shown to have a distinct gender bias. For example, idiopathic PF is more common in males (Brass et al., 2010; Carey, Card, Voltz, Arbes, et al., 2007; Iwai et al., 1994) and women have been shown to have better outcomes in this disease than men (Gribbin et al., 2006). Investigations of silica-induced PF from our laboratory show that male mice are more sensitive to this insult (Brass et al., 2010). In addition, silica-exposed female mice developed less lung fibrosis but have more lung inflammation compared to males.

Moreover, ovariectomized female mice show inflammatory and fibrotic responses similar to males, suggesting that estrogen may protect against silica-induced pulmonary fibrosis (Brass et al., 2010).

Neutrophils are the first responders to infection and tissue injury (Jennings & Knaus, 2014), including injury due to silica exposure (Hornung et al., 2008). Upon inhalation, silica particles are phagocytized by neutrophils and macrophages recruited into the lungs. Silica-laden neutrophils and macrophages have a reduced capacity to clear additional silica particles by phagocytosis. Therefore, the persistence of silica particles in the lung may drive the continuous influx of inflammatory cells (Adamson & Bowden, 1984). Silica particles induce inflammatory responses by activating the NALP3 inflammasome (Cassel et al., 2008; Ramsgaard et al., 2010) to produce IL-1β. Interestingly, neutrophil influx is mediated by IL-1β, which is produced in higher amounts in silica-exposed mice (Hornung et al., 2008).

A gender bias has been observed in neutrophil recruitment to sites of injury. Women and female animals recruit greater numbers of neutrophils compared to males in instances of alcoholic acute liver injury (Eagon, 2010) and neutrophil counts are higher in females of all ages under severe acute inflammatory conditions (Casimir et al., 2013).

The recruitment of neutrophils to the site of injury involves multiple steps from chemotaxis to cellular adhesion and trans-endothelial migration (TEM) (Basit et al., 2006; Greenberger et al., 1996; Ochietti et al., 2002; L. Yang et al., 2005). CD11/CD18-dependent and-independent pathways mediate neutrophil adherence to pulmonary microvascular endothelial cells and migration to the distal spaces of the lungs (Doerschuk et al., 2000; Long, 2011). IL-1 (Hornung et al., 2008) and TNFα (Vanhee et al., 1995),

which are induced in silicosis and mouse model of silica-induced PF, activate the CD11/CD18 dependent pathway, leading to the production of ICAM-1 (Doerschuk et al., 2000; Long, 2011; Mizgerd, 2002). ICAM-1 is a critical mediator of neutrophil adhesion, recruitment and TEM to sites of injury (Basit et al., 2006; Ochietti et al., 2002; L. Yang et al., 2005). ICAM-1 is constitutively expressed on lung endothelial and epithelial cells where it mediates TEM of neutrophils and retention of neutrophils at the epithelial surface, respectively (Nario & Hubbard, 1996). ICAM-1 is upregulated by a number of different cytokines, chemokines and growth factors. For example, IL-1 and TNFα can induce ICAM-1 expression and interestingly, theses cytokines are upregulated in BAL of both silicosis patients (Vanhee et al., 1995) and silica-exposed mice (Ohtsuka et al., 1995). In addition, MIP-2 is a potent chemotactic factor for neutrophils in lung inflammation (Driscoll, 1994) and is one of several cytokines involved in mediating neutrophillic inflammation following a single instillation of crystalline silica (Yuen et al., 1996). NF-kB, which is induced by silica (Di Giuseppe et al., 2009), also induces the expression of ICAM-1 and MIP-2 and, by extension, neutrophil recruitment to the lungs following bacterial infection in rodents (Mizgerd, 2002). Furthermore, human neutrophils express VEGF and neutrophil influx is associated with increased vascular permeability, which is the hallmark of acute lung injury (Kolaczkowska & Kubes, 2013; van Der Flier, Coenjaerts, Kimpen, Hoepelman, & Geelen, 2000; Webb, Myers, Watson, Bottomley, & Brenchley, 1998). In addition, VEGF upregulates ICAM-1 in brain microvascular endothelial cells (BMEC) (Radisavljevic, Avraham, & Avraham, 2000).

The matricellular protein secreted phosphoprotein 1 (SPP1) exhibits both pro-inflammatory and anti-inflammatory effects (Lund et al., 2009) depending upon the

cellular context (L. Bird, 2007; Xanthou et al., 2007). SPP1 plays a role in many biological and pathological processes including, but not limited to: developmental processes, wound healing, fibrosis, immunological responses, tumorigenesis and bone resorption and calcification (Sodek et al., 2000). SPP1 mediates cell adhesion, migration, tumor invasion, has T- helper 1 (Th1) cytokine function, and anti-apoptotic effects via interaction with integrins and CD44 (Buback et al., 2009; Denhardt, Noda, et al., 2001). SPP1 plays a critical role in the pathogenesis of IPF and animal models of pulmonary fibrosis. For example, SPP1 expression is significantly elevated in bleomycin-induced pulmonary fibrosis in mice (Takahashi et al., 2001b). Oligonucleotide array showed that SPP1 is also elevated in the lungs of IPF patients relative to normal lungs and increased in the BALF of IPF patients (Pardo et al., 2005). Data from our laboratory show that SPP1 protein is differentially expressed in male and female mice at 14 days post-silica exposure with males showing higher BALF and tissue-associated SPP1 compared to females. We have also demonstrated that estrogen reduced the levels of secreted SPP1 in male mice (Fig: 6). The role of SPP1 in neutrophil recruitment is controversial. SPP1 augments neutrophil recruitment in Klebsiella-induced pneumonia (van der Windt et al., 2010), but it does not influence neutrophil recruitment in pneumococcal pneumonia (van der Windt et al., 2011).

In this study, we hypothesized that female mice recruit greater numbers of neutrophils at pre-fibrotic time points and that the SPP1 regulation of ICAM-1 mediates the gender-specific differences in neutrophil recruitment to the lungs following exposure to crystalline silica. We have shown here that SPP1 regulation of ICAM-1 does not support the gender-specific difference in neutrophil recruitment at pre-fibrotic time points following silica exposure in mice.

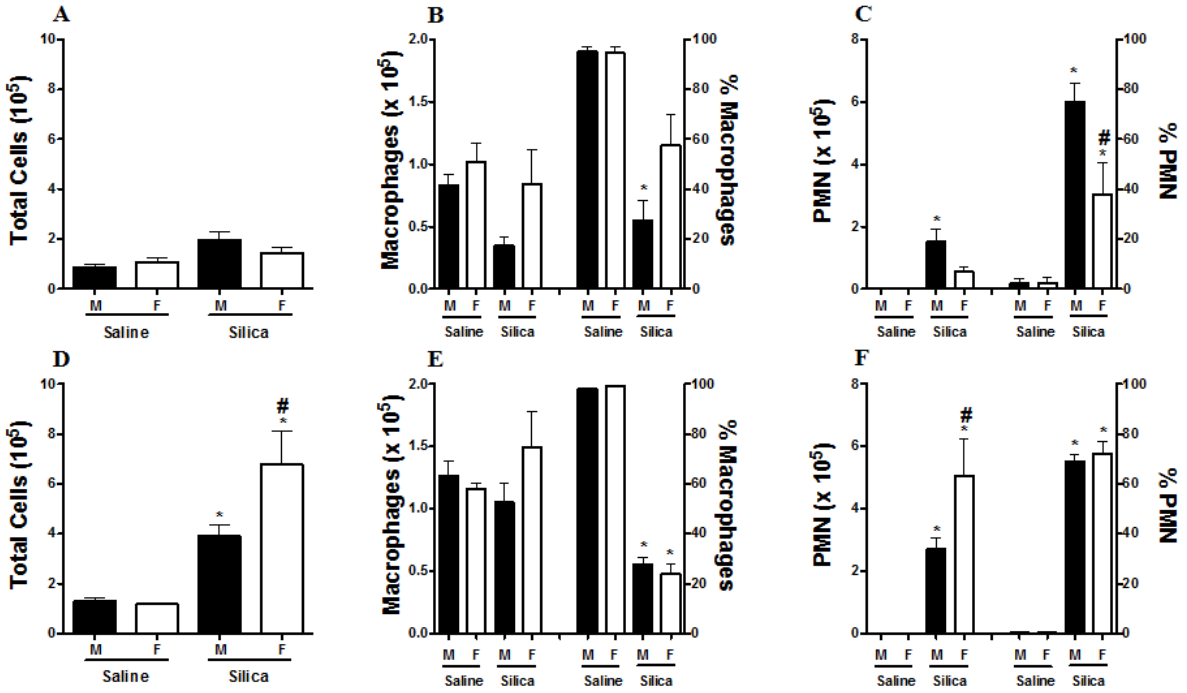
3.3 RESULTS

Silica-exposed female mice recruit greater numbers of neutrophils compared to silica-exposed males at 3 days post-treatment.

Our laboratory has previously shown that silica-exposed female mice recruit greater numbers of inflammatory cells to the lung compared to silica-exposed male mice at 14 days post-treatment (Brass et al., 2010).

To determine if there are gender-specific differences in the silica-induced inflammatory response at early pre-fibrotic time points, we intratracheally exposed 8-10 week old C57BL/6J male and female mice to 0.2 mg/kg body weight of freshly fractured crystalline silica. We assessed inflammation at 24 h and 3 days post-silica exposure in BAL fluid and lung tissue by differential analysis of Protocol Hema 3-stained inflammatory cells and Masson's trichrome-stained histological sections, respectively. BALF analysis (Fig 8) shows that silica-exposed mice of either gender recruit greater total numbers of inflammatory cells, as compared to saline-exposed control mice. Inflammatory cell recruitment is higher after 3 days of exposure compared to 24 h (Fig: 8 D vs. A). In addition, there are significantly greater numbers of BALF inflammatory cells in silica-exposed female mice compared to silica-exposed males after 3 days, but not after 24 h, of exposure (Fig: 8 D vs. A). Contrary to earlier observations (Brass et al., 2010), significant differences in macrophage recruitment between the silica-exposed mice and the saline-exposed controls or between the silica-exposed female and male mice at either 24 h or 3 days post-treatment were not observed (Fig 8 E vs. B). However, neutrophil numbers were significantly increased in the silica-exposed mice compared to the saline-

exposed controls at 3 days, but not 24 h, post-treatment (Fig: 8 F vs. C). Interestingly, silica-exposed female mice recruit greater numbers of neutrophils compared to silica-exposed males at 3 days post-silica exposure (Fig: 8F).

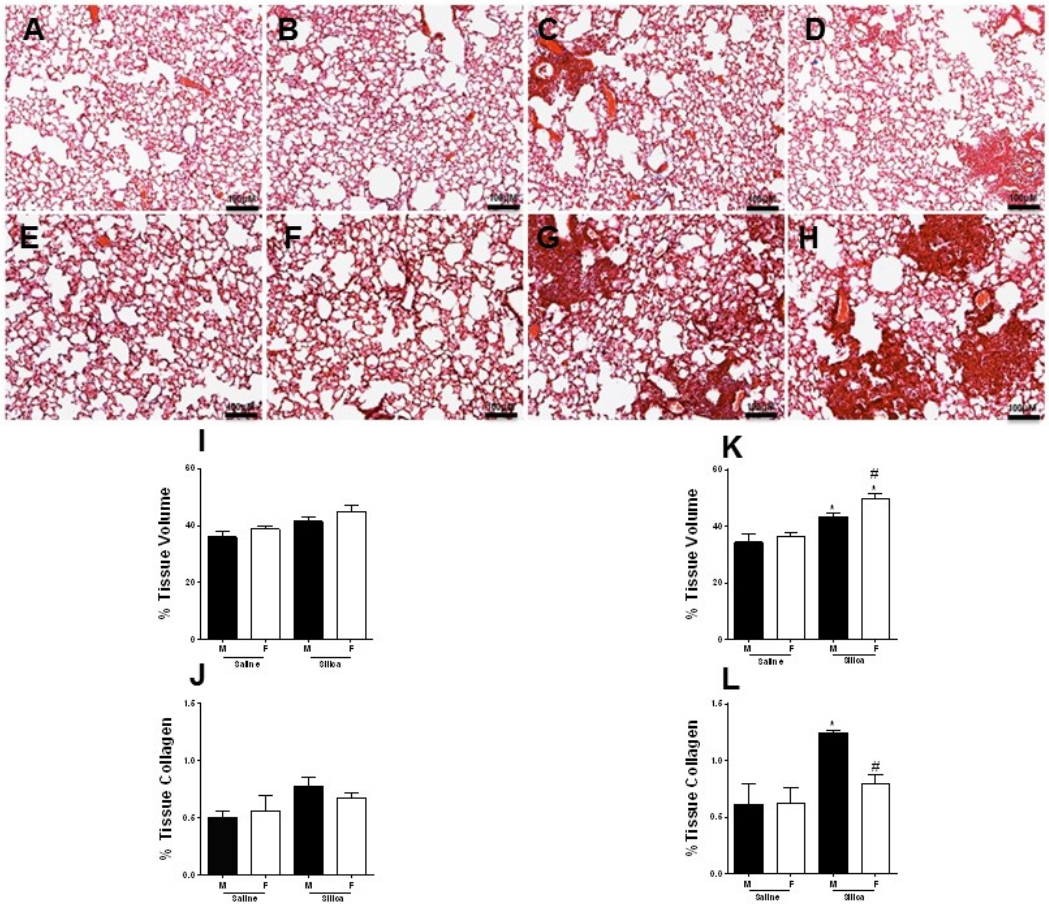


This panel shows the total number of inflammatory cells from whole lung lavage fluid at 24 h (A) and 3 days (D) post-silica exposure. The number and percentage of total inflammatory cells that are macrophages at 24 h (B) and at 3 days (E) post-silica exposure are shown. The number and percentage of total inflammatory cells that are neutrophils at 24 h (C) and at 3 days (F) post-silica exposure are also depicted. N ≥ 3 per group. * indicates $p<0.05$ vs. same-gender saline, # indicates $p<0.05$ vs. silica-exposed male mice, by one-way ANOVA followed by Bonferroni correction for multiple comparisons.

Figure 8: Silica-exposed female mice recruit greater numbers of neutrophils compared to silica-exposed males at 3 days post-treatment.

Tissue injury is increased in the lungs of silica-exposed female mice compared to exposed male mice at pre-fibrotic time points.

Figure 9 shows that at 24 h post-silica exposure, the amount of tissue injury was not significantly different between saline-and silica-exposed mice (Fig: 9 A and B vs. C and D and I) or between silica-exposed male and female mice (Fig: 9 C vs. D, and I). However, at 3 days post-exposure there is a significant difference in tissue injury between the silica-exposed mice and the saline-exposed controls (Fig: 9 G vs. E, H vs. F, and K). Furthermore, silica-exposed female mice show a greater degree of tissue injury compared to exposed males at 3 days post-treatment (Fig: 9 H vs. G, and K). Finally, silica-exposed female mice have less lung collagen compared to exposed males at 3 days, but not at 24 h, post-silica exposure (Fig: 9 H vs. G, D vs. C, L, and J).



The upper panel shows Masson's trichrome staining of lung tissue from (A) saline-exposed male, (B) saline-exposed female, (C) silica-exposed male, (D) silica-exposed female mice at 24 h post-exposure. The middle panel shows trichrome stained tissue sections from (E) saline-exposed male, (F) saline-exposed female, (G) silica-exposed male, (H) silica-exposed female mice at 3 days post-exposure. The bottom panel shows (I) percentage tissue volume and (J) percentage collagen at 24 h post-exposure. (K) the percentage tissue volume and (L) percentage collage at 3 days post-exposure. N ≥ 3 per group. * indicates $p<0.05$ vs. same-gender saline, # indicates $p<0.05$ vs. silica-exposed male mice by one-way ANOVA followed by Bonferroni correction for multiple comparisons.

Figure 9: Tissue injury is increased in the lungs of silica-exposed female mice compared to exposed male mice at pre-fibrotic time points.

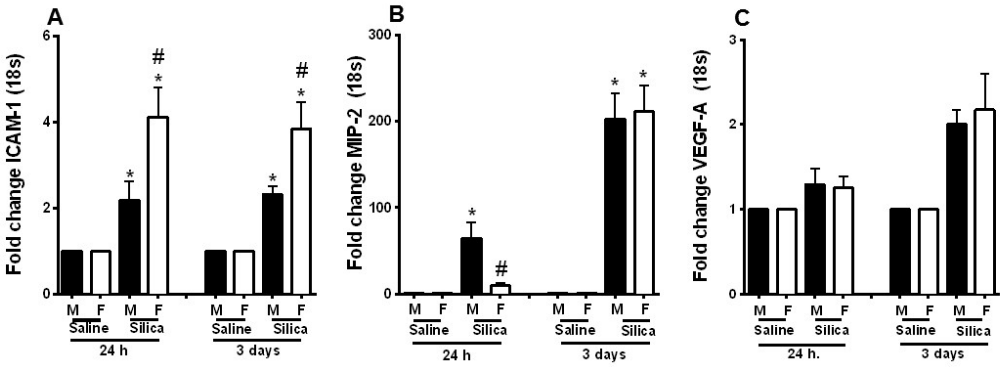
ICAM-1 mRNA expression is increased in silica-exposed female mice at pre-fibrotic time points.

The recruitment of neutrophils to the site of injury involves multiple steps from chemotaxis to cellular adhesion and trans-endothelial migration (TEM) (Basit et al., 2006; Greenberger et al., 1996; Ochietti et al., 2002; L. Yang et al., 2005). ICAM-1 is known to be critical for neutrophil recruitment, as this molecule regulates neutrophil adhesion and TEM to sites of injury (Basit et al., 2006; Ochietti et al., 2002; L. Yang et al., 2005).

To determine the effects of silica exposure on the expression of ICAM-1 and other neutrophil recruitment factors, we measured the relative mRNA levels of ICAM-1, MIP-2, and VEGF-A by RT-PCR in lung tissue from male and female mice exposed to silica for either at 24 h or 3 days.

Figure 10 shows that silica-exposed female mice express higher levels of ICAM-1 mRNA compared to exposed males at both 24 h and 3 days post-silica exposure (Fig: 10A). In addition, MIP-2 mRNA expression is reduced in the lungs of silica-exposed female mice compared to exposed males at 24 h post-treatment (Fig: 10B). However, MIP-2 mRNA expression is not significantly different between silica-exposed male and female mice at 3 days post-treatment (Fig: 10B).

Finally, a significant difference was not observed in VEGF-A mRNA expression between silica-exposed male and female mice at either 24 h or 3 days post-treatment (Fig: 10C).



This panel shows the mRNA levels for (A) ICAM-1 (B) MIP-2 and (C) VEGF-A at 24 h and 3 days post-silica exposure. N ≥ 3per group. * indicates $p<0.05$ vs. same-gender saline, # $p<0.05$ vs. silica exposed male mice by one-way ANOVA followed by the Bonferroni correction for multiple comparisons.

NB: mRNA levels in silica-exposed mice were normalized to the respective saline-exposed controls and to Rn18s as an endogenous control.

Figure 10: ICAM-1 mRNA expression is increased in silica-exposed female mice at pre-fibrotic time points.

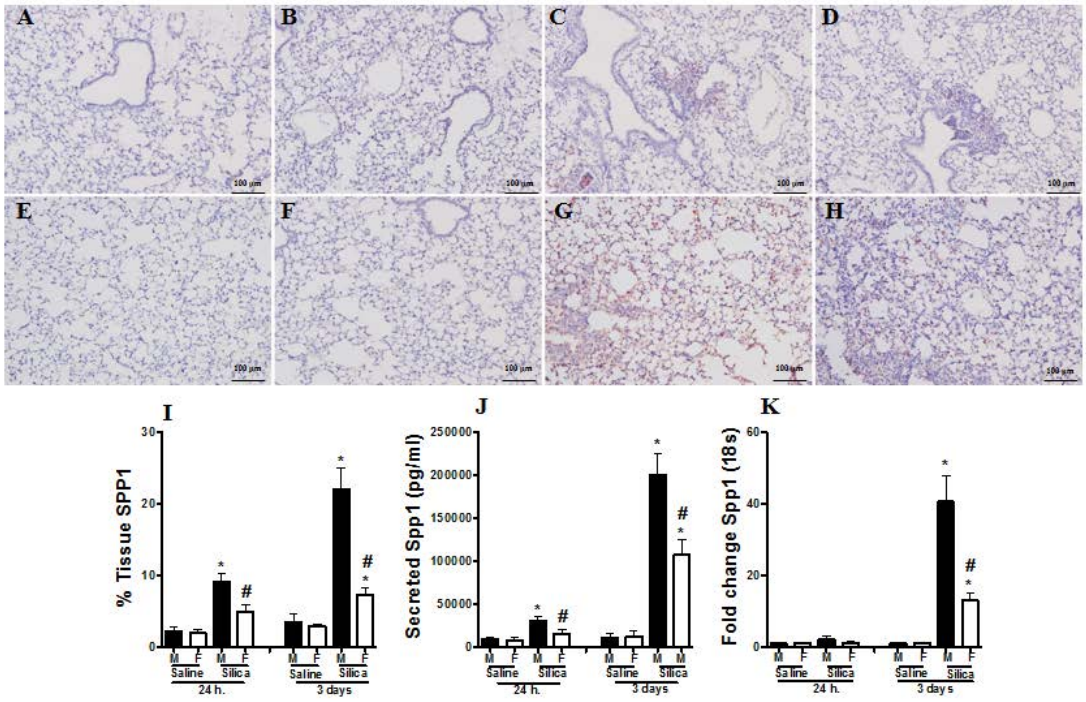
Silica-exposed female mice express less SPP1 mRNA and protein than exposed males at pre-fibrotic time point.

SPP1 is upregulated in numerous animal models of pulmonary fibrosis (Sabo-Attwood et al., 2011; Takahashi et al., 2001a), as well as in idiopathic pulmonary fibrosis (Pardo et al., 2005) and silicosis (Nau et al., 1997) patients. We also have shown that SPP1 is upregulated in our mouse model of silica-induced pulmonary fibrosis and that silica-exposed female mice express less lung SPP1 mRNA and protein compared to males at 14 days post-treatment.

To assess the expression of SPP1 and to determine if there is a gender-based difference in SPP1 expression at pre-fibrotic time points, we exposed mice to silica as described above and assessed the levels of tissue-associated SPP1, secreted SPP1 and SPP1 mRNA by immunohistochemistry, ELISA and RT-PCR, respectively.

Silica-exposed male but not exposed female mice express greater levels of tissue-associated SPP1 compared to saline-exposed control mice at 24 h post-treatment (Fig: 11 C vs. A, D vs. B, and I). Silica-exposed female mice express less tissue-associated SPP1 compared to silica-exposed males at 24 h post-treatment (Fig: 11 D vs. C, and I). At 3 days post-treatment, silica-exposed male and female mice express higher levels of tissue-associated SPP1 compared to the respective saline-exposed controls (Fig 11 G vs. E, H vs. F, and I). Furthermore, silica-exposed females express less tissue-associated SPP1 compared to males at this time point (Fig: 11 H vs. G, and I). Silica-exposed male but not exposed female mice express greater levels of secreted SPP1 compared to saline-exposed controls at 24 h post-treatment (Fig: 11J). Silica-exposed female mice express less secreted SPP1 compared to exposed males at 24 h post-treatment (Fig:

11J). At 3 days post-silica exposure, male and female mice express higher levels of secreted SPP1 compared to the respective saline-exposed controls (Fig: 11J). Furthermore, silica-exposed females express less secreted SPP1 compared to exposed males at 3 days post-treatment (Fig: 11J).



The upper panel shows the results of immunohistochemical staining for SPP1 in lung tissue from (A) saline-exposed male, (B) saline-exposed female, (C) silica-exposed male, (D) silica-exposed female mice at 24 h post-silica exposure. The middle panel shows immunohistochemical staining for SPP1 in lung tissue from (E) saline-exposed male, (F) saline-exposed female, (G) silica-exposed male, (H) silica-exposed female mice at 3 days post-silica exposure. The percentage of tissue staining for SPP1 at 24 h and 3 days post silica-exposure are shown in (I). The levels of secreted SPP1 in whole lung lavage at 24 h and 3 days post-silica exposure are shown in (J). The levels of SPP1 mRNA in lung tissue at 24 h and 3 days post-silica

exposure are shown in (K). N ≥ 3per group. * indicates $p<0.05$ vs. same-gender saline, # indicates $p<0.05$ vs. silica-exposed male mice by one-way ANOVA followed by the Bonferroni correction for multiple comparisons. NB: RT-PCR results from silica-exposed mice were normalized against the respective saline-exposed controls and Rn18s as an endogenous control.

Figure 11: SPP1 is reduced in silica-exposed female mice compared to males at pre-fibrotic time points.

At 24 h post-exposure, SPP1 mRNA expression is not significantly different between the silica-exposed mice and the saline-exposed controls (Fig: 11K). In addition, SPP1 mRNA levels are not different between silica-exposed male and female mice at 24 h post-exposure (Fig: 11K).

However, silica-exposed male and female mice express greater levels of SPP1 mRNA compared to the respective saline-exposed controls at 3 days post-treatment (Fig: 11K). Finally, silica-exposed female mice express less SPP1 mRNA compared to exposed males at 3 days post-treatment (Fig: 11K).

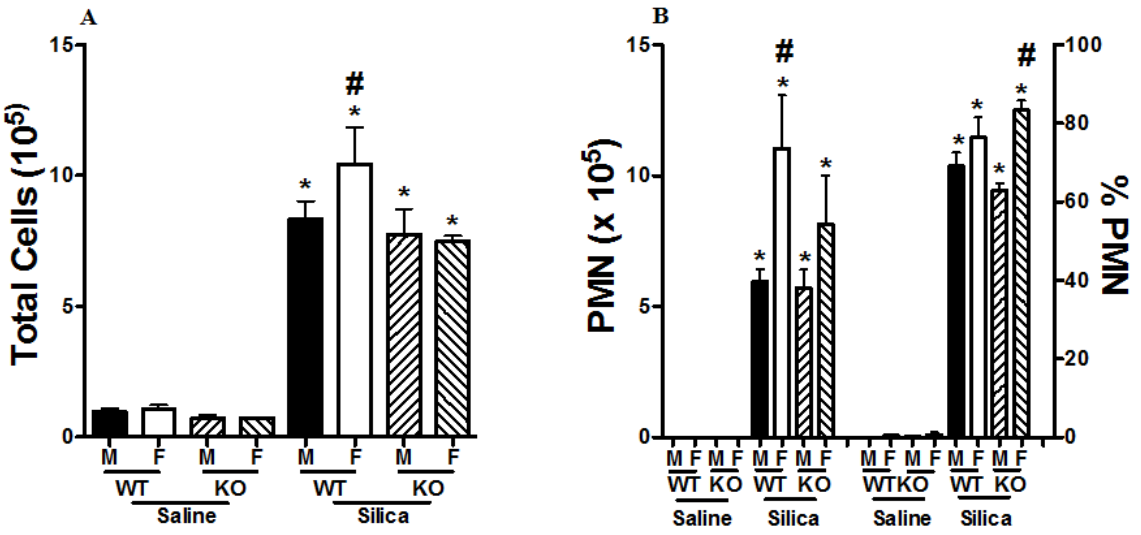
SPP1 deficiency does not influence neutrophil recruitment and tissue injury in mice at 3 days post-silica exposure.

SPP1 is known to act as a pro-inflammatory cytokine (Denhardt, Giachelli, et al., 2001; Lund et al., 2009; Mazzali et al., 2002; M. Yang et al., 2014) but exhibits anti-inflammatory properties under certain conditions (Denhardt, Giachelli, et al., 2001; Lund et al., 2009).

To determine if SPP1 plays an important role in inflammatory responses at pre-fibrotic time points, we exposed C57BL/6J wild type and SPP1-deficient male and female mice to silica as described above and assessed inflammation in BALF and lung tissue by

differential analysis of Protocol Hema 3-stained inflammatory cells and Masson's trichrome-stained histological sections, respectively, at 3 days post-silica exposure.

BALF analysis (Figure 12) shows that silica-exposed SPP1-deficient mice recruit greater total numbers of inflammatory cells compared to saline-exposed controls (Fig: 12A). The total numbers of inflammatory cells are not significantly different between silica-exposed SPP1-deficient female and male mice (Fig: 12A). Furthermore, the total numbers of inflammatory cells are not significantly different between either silica-exposed SPP1-deficient and wild type female mice, or between silica-exposed SPP1-deficient and wild type male mice at 3 days post-treatment (Fig: 12A). Interestingly, there is a significant increase in neutrophils in silica-exposed SPP1-deficient mice compared to saline-exposed controls (Fig: 12B). However, the number of neutrophils is not significantly different between silica-exposed SPP1-deficient female and male mice (Fig: 12B). In addition, the number of neutrophils is not significantly different between silica-exposed SPP1-deficient and wild type female mice or between silica-exposed SPP1-deficient and wild type male mice at 3 days post-treatment (Fig: 12B).

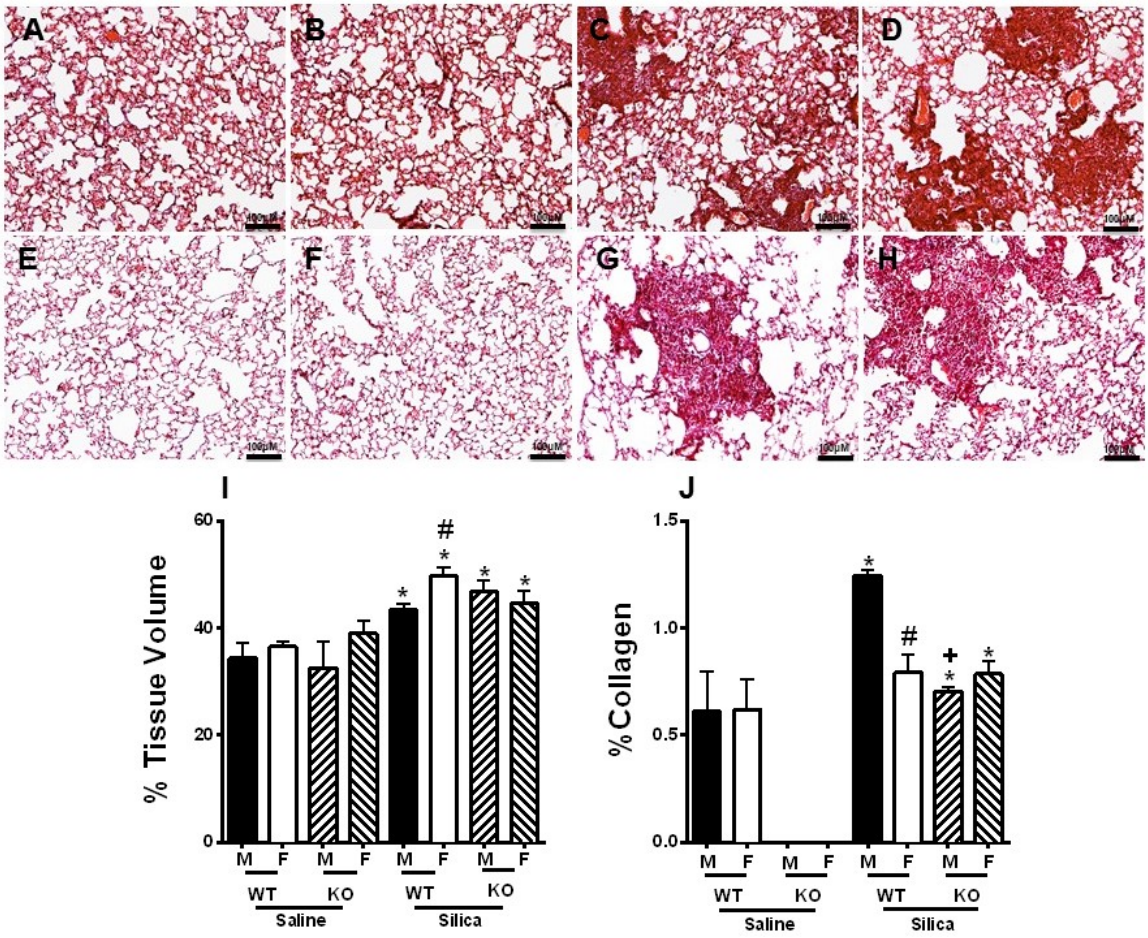


This panel shows total numbers of inflammatory cells (A) in whole lung lavage from saline and silica-exposed wild type (WT) and SPP1-deficient (KO) mice at 3 days post-silica exposure. The number and percentage of total inflammatory cells that are neutrophils (B) are shown. N ≥ 3per group. * indicates $p<0.05$ vs. same-gender saline, # indicates $p<0.05$ vs. silica exposed male mice by one-way ANOVA followed by Bonferroni correction for multiple comparisons.

Figure 12: Loss of SPP1 does not influence neutrophil recruitment in mice at 3 days post-silica exposure.

Figure 13 shows that the extent of disease injury in the lung tissue of silica-exposed SPP1-deficient mice is greater than in the saline-exposed controls (Fig: 13 G vs. E, H vs. F, and I). The amount of involved tissue is not significantly different between silica-exposed SPP1-deficient female and male mice (Fig: 13 H vs. G, and I). In addition, the

amount of involved tissue is not significantly different between silica-exposed SPP1-deficient and wild type female mice (Fig: 13 H vs. D, and I) or between silica-exposed SPP1-deficient and wild type male mice (Fig: 13 G vs. C, and I), at 3 days post-treatment. In addition, the amount of collagen deposition is not significantly different between silica-exposed SPP1-deficient female and male mice (Fig: 13 H vs. G, and J). However, silica-exposed SPP1-deficient mice show significant increases in tissue collagen compared to the saline-exposed controls (Fig: 13 G vs. E, H vs. F, and J). Silica-exposed SPP1-deficient male mice show a significant decrease in tissue collagen compared to wild type males (Fig: 13 G vs. C, and J) but these differences were not observed in the females (Fig: 13 H vs. D, and J).



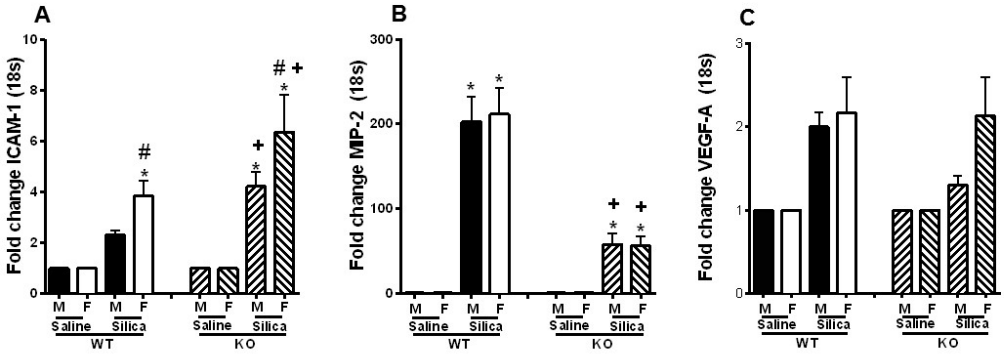
The upper panel shows Masson's trichrome staining of lung tissue from (A) saline-exposed male, (B) saline-exposed female, (C) silica-exposed male, (D) silica-exposed female wild type (WT) mice at 3 days post-exposure. The middle panel shows tissue sections from (E) saline-exposed male, (F) saline-exposed female, (G) silica-exposed male, (H) silica-exposed female SPP1-deficient (KO) mice at 3 days post-silica exposure. The bottom panel shows (I) percent tissue volume and (J) percent tissue collagen at 3 days post-silica exposure. N ≥ 3per group. * indicates $p<0.05$ vs. same-gender saline, # indicates $p<0.05$ vs. silica-exposed male mice, + indicates $p<0.05$ vs. wild type mice by one-way ANOVA followed by Bonferroni correction for multiple comparisons. NB: figure 9 E, F, G, and F is similar to figure 13 A, B, C and D respectively.

Figure 13: Loss of SPP1 does not influence lung injury at 3 days post-silica exposure.

Loss of SPP1 upregulates ICAM-1 and downregulates MIP-2 at 3 days post-silica exposure.

To further investigate the role of SPP1 in regulation of silica-induced neutrophil recruitment at pre-fibrotic time points, we exposed mice to silica as described above and determined the relative mRNA levels of ICAM-1, MIP-2 and VEGF-A by RT-PCR at 3 days post-treatment.

Figure 14 shows that silica-exposed wild type and SPP1-deficient female mice express greater levels of ICAM-1 mRNA compared to the respective wild type and SPP1-deficient males (Fig: 14A). Silica-exposed SPP1-deficient female and male mice express greater levels of ICAM-1 mRNA compared to same gender wild type mice (Fig: 14A). The levels of MIP-2 mRNA expression are reduced in silica-exposed SPP1-deficient mice compared to wild type mice (Fig: 14B). However, MIP-2 mRNA levels are not significantly different between silica-exposed SPP1-deficient female and male (Fig: 14B). Finally, the VEGF-A mRNA expression between neither the silica-exposed SPP1-deficient mice and wild type nor silica-exposed SPP1-deficient female and male mice is significantly different (Fig: 14C).



This panel shows mRNA expression levels for (A) ICAM-1 (B) MIP-2 and (C) VEGF-A in wild type (WT) and SPP1-deficient (KO) mice at 3 days post-silica exposure. N ≥ 3 per group, * indicates $p<0.05$ vs. same-gender saline, # indicates $p<0.05$ vs. silica-exposed male mice, + indicates $p<0.05$ vs. wild type mice by one-way ANOVA followed by Bonferroni correction for multiple comparisons. NB: mRNA levels in silica-exposed mice were normalized to mRNA levels in the respective saline control mice and to Rn18s as an endogenous control.

Figure 14: Loss of SPP1 upregulates ICAM-1 and downregulates MIP-2 at 3 days post-silica exposure.

3.4 DISCUSSION

In this study, we focused on the role of gender and SPP1 in silica-induced lung neutrophil recruitment and early disease-related tissue changes at pre-fibrotic time points. We demonstrate that female mice recruit a greater numbers of inflammatory cells, the majority of which are neutrophils, and show a greater extent of lung tissue injury compared to male mice at pre-fibrotic time points. Silica-exposed female mice express greater levels of ICAM-1 mRNA compared to exposed males at pre-fibrotic time points. In addition, we have shown that female mice express less SPP1 mRNA and secrete less SPP1 protein compared to male mice at the same time points. Here, we show that SPP1 deficiency does not influence neutrophil recruitment and lung tissue damage at 3 days post-silica treatment. Finally, SPP1 deficiency upregulates ICAM-1 mRNA expression, however, SPP1-mediated downregulation of ICAM-1 does not support the gender-specific differences in neutrophil recruitment at pre-fibrotic time points.

Inhalation of crystalline silica causes an inflammatory reaction characterized by the infiltration of macrophages and neutrophils into the lungs (Y. Chen et al., 2013; Costantini, Gilberti, & Knecht, 2011; Sato et al., 2008) and the production of pro-inflammatory cytokines, chemokines and reactive oxygen species (ROS) (Sato et al., 2008). Upon inhalation, silica particles are phagocytized by recruited neutrophils and macrophages. One of the driving forces behind the influx of inflammatory cells is the decreased phagocytic capacity of silica-laden neutrophils and macrophages, leading to an increase in cell numbers available to provide clearance (Adamson & Bowden, 1984). At 14 days post-silica exposure, macrophages are the predominant inflammatory cell present in the lungs (Brass et al., 2010; Ramsgaard et al., 2010). However, at earlier time

points following silica exposure, neutrophils are the predominant inflammatory cells (Nario & Hubbard, 1997; Sato et al., 2008). Consistent with the above information, we show that neutrophils are the predominant inflammatory cell type recruited into the lungs at 3 days post-silica exposure (Fig: 8F). Previous results from our laboratory show that female mice are protected against development of silica-induced pulmonary fibrosis and recruit more inflammatory cells, predominantly macrophages, at 14 days post-exposure (Brass et al., 2010).

Other groups have also observed a gender bias in immune cell recruitment. For example, female rats and mice recruit fewer neutrophils to sites of burn injury compared to males (M. D. Bird et al., 2008). Contrary to this, women and female animals with alcoholic acute liver injury recruit a greater numbers of neutrophils compared to males (Eagon, 2010) and neutrophil counts are reported to be higher in females of all ages in severe acute inflammatory conditions (Casimir et al., 2013). It is known that estrogen increases neutrophil survival (Molloy et al., 2003) and significantly increases lung inflammation and neutrophil recruitment in *Pseudomonas aeruginosa* pneumonia in a mouse model of cystic fibrosis (Y. Wang et al., 2010), but suppresses neutrophil recruitment in LPS-induced acute lung injury in mice (Speyer et al., 2005). Taken together, this information shows that the effects of gender on inflammation is ambiguous and depends upon both the duration of exposure and the nature of the injury. In this study, we demonstrate that silica-exposed female mice recruit significantly greater numbers of total inflammatory cells and neutrophils compared to exposed male mice at 3 days post-treatment (Fig: 8 D and F). It is known that the activation and migration of neutrophils into the lungs contributes to inflammatory tissue injury and remodeling of tissue architecture

(Wagner & Roth, 2000). In view of this, we demonstrate that female mice show greater tissue injury compared to male mice at 3 days post-silica exposure (Fig: 9 H vs. G, and K).

Numerous studies indicated that ICAM-1 is critical for neutrophil recruitment to sites of injury (Basit et al., 2006; Nario & Hubbard, 1996; Ochietti et al., 2002; Pang, Hong, West-Barnette, Kock, & Swords, 2008; L. Yang et al., 2005) and plays a role in silica-induced neutrophil recruitment in mice (Hubbard, Thibodeau, & Giardina, 2001; Nario & Hubbard, 1996). A 4 h exposure to estrogen increases tumor necrosis factor-induced ICAM-1 expression in human umbilical vein endothelial cells, but a 6 h exposure has the opposite effect (Cid et al., 1994). Here we demonstrate that silica-exposed female mice express greater levels of ICAM-1 mRNA compared to exposed male mice at both 24 h and 3 days post-treatment (Fig: 10A). Therefore, ICAM-1 plays a role in the observed gender difference in neutrophil recruitment at both 24 h and 3 days post-silica exposure. MIP-2 is a potent chemotactic factor for neutrophils in lung inflammation (Driscoll, 1994) and is one of several cytokines involved in mediating neutrophillic inflammation following a single instillation of crystalline silica (Yuen et al., 1996). Our results show that female mice express less MIP-2 mRNA compared to male mice at 24 h post-silica exposure. However, at 3 days post-exposure MIP-2 mRNA expression is increased compared to 24 h but MIP-2 expression is not significantly different between male and female mice at this time point (Fig: 10B).

Human neutrophils express VEGF and neutrophil influx is associated with increased vascular permeability, which is the hallmark of acute lung injury (Kolaczkowska & Kubes, 2013; van Der Flier et al., 2000; Webb et al., 1998). Here we show that VEGF-

A mRNA expression is not significantly different between female and male mice at both 24 h and 3 days post-silica exposure (Fig: 10C). These results indicate that both MIP-2 and VEGF-A do not play a role in the observed gender differences in neutrophil recruitment and lung injury at 3 days post-silica exposure.

SPP1 is upregulated in numerous animal models of pulmonary fibrosis (Sabo-Attwood et al., 2011; Takahashi et al., 2001a), as well as in idiopathic pulmonary fibrosis (Pardo et al., 2005) and silicosis (Nau et al., 1997) patients. Interestingly, estrogen inhibits SPP1 production in vascular smooth muscle cells (Li et al., 2000). In a rodent model of alcoholic steatohepatitis, low doses of estrogen downregulate the expression of SPP1, while high doses of estrogen upregulate SPP1 expression (Banerjee et al., 2009). Neutrophils secrete low levels of SPP1 compared to macrophages (Koh et al., 2007) and earlier we showed that female mice express less SPP1 compared to male mice at 14 days post-silica exposure (unpublished data). Estrogen treatment of male mice reduced SPP1 expression at 14 days post-silica exposure (see Figure 6). In support of the above findings, we demonstrate that silica-exposed female mice secret and express less SPP1 than exposed males at both 24 h and 3 days post-silica exposure (Fig: 11 H vs. G, J and I). In addition, silica-exposed female mice express less SPP1 mRNA compared to exposed males at 3 days, but not 24 h post-treatment (Fig: 11K). This indicates that gender-specific difference in SPP1 expression might play a role in the observed gender difference in neutrophil recruitment and lung injury at pre-fibrotic time points.

SPP1 exhibits pro-inflammatory and anti-inflammatory effects (Lund et al., 2009) and can have opposing effects depending on cellular context (L. Bird, 2007; Xanthou et al., 2007). Furthermore, SPP1 plays a role in bleomycin-induced pulmonary fibrosis

(Berman et al., 2004) and our laboratory has shown that SPP1 is important in silica-induced pulmonary fibrosis in mice (unpublished data). SPP1 is chemotactic to neutrophils (van der Windt et al., 2010) but SPP1 deficiency does not affect the phagocytic ability or the generation of reactive oxygen species by neutrophils (Koh et al., 2007). In addition, SPP1 deficiency does not influence neutrophil recruitment in response to streptococcus pneumonia (van der Windt et al., 2011).

Consistent with these studies, we showed that the total number of inflammatory cells and neutrophils are not affected by SPP1 deficiency and the gender differences are abolished in SPP1-deficient mice at 3 days post-silica exposure (Fig: 12 A and B). We also showed that overall tissue pathology is not affected by SPP1 deficiency (Fig: 13 G vs. C, H vs. D, H vs. G, and I). The above findings do not support part of our hypothesis that SPP1 plays a role in the gender-specific difference in neutrophil recruitment after silica exposure at pre-fibrotic time points. Estrogen may be a possible driving force in this phenomenon as previous studies have shown that estrogen significantly increase neutrophil recruitment (Robinson, Hall, Nilles, Bream, & Klein, 2014; Y. Wang et al., 2010). We have previously showed that estrogen increases neutrophil recruitment at 14 days post-silica treatment in mice (Fig: 3D). Furthermore, physiological levels of estrogen delay apoptosis of neutrophils in both men and women (Molloy et al., 2003). SPP1 is known to enhance neutrophil recruitment by binding to ITGA9B1 and ITGA4B1 integrins present on neutrophils (Banerjee et al., 2008; Barry, Ludbrook, Murrison, & Horgan, 2000). The fact that SPP1 deficiency does not influence neutrophil recruitment suggests that SPP1 is chemoattractant to neutrophils as previously reported by others (Barreno et al., 2013; van der Windt et al., 2010) (Barreno et al., 2013; van der Windt et al., 2010) but

does not play a role in the gender-specific difference in neutrophil recruitment at profibrotic time points in mice. An earlier study showed that naive adult male C57BL/6 mice had 25% more lung hydroxyproline compared to age-matched females (Carey, Card, et al., 2007b). Here we show that SPP1 deficiency reduces tissue collagen deposition in male but not female mice, and that the gender difference in collagen deposition is abolished at 3 days post-silica exposure (Fig: 13 G vs. C, H vs. D, H vs. G, and J). This shows that SPP1 is a pro-fibrotic molecule, as has been previously demonstrated in our laboratory and by others.

SPP1 has been shown to induce the expression of ICAM-1 in human MCF-7 and MDA MB-468 breast cancer cells (Ahmed & Kundu, 2010). Other studies have demonstrated that SPP1 does not affect MIP-2 expression in response to Streptococcal pneumonia (van der Windt et al., 2011) and Klebsiella pneumonia in mice (van der Windt et al., 2010). On the contrary, we show here that SPP1 deficiency upregulates ICAM-1 mRNA expression, while MIP-2 mRNA expression is downregulated in both male and female mice at 3 days post-silica exposure (Fig: 14 A and B). Female SPP1-deficient mice express greater levels of ICAM-1 mRNA than male mice, but significant differences in MIP-2 expression were not observed between genders at 3 days post-silica exposure (Fig: 14 A and B). Furthermore, SPP1 augments the expression of VEGF in MDA-MB-231 cells (Chakraborty, Jain, & Kundu, 2008) and VEGF increases SPP1 expression in HUVEC (Infanger et al., 2008). However, we observe that SPP1-deficiency does not affect the expression levels of VEGF-A mRNA in either male or female mice at 3 days post-silica exposure (Fig 14C). The upregulation of ICAM-1 mRNA in SPP1 deficient mice does not show a corresponding increase in the numbers recruited neutrophil in response

to silica at pre-fibrotic time points. Therefore, upregulation of ICAM-1 may not reflect an increase in ICAM-1 protein in SPP1 deficient mice. In view of fact that male mice express greater levels of SPP1 and loss of SPP1 upregulates ICAM-1 mRNA, SPP1-mediated suppression of ICAM-1 does not explain the reduced neutrophil recruitment in male mice compared to female mice at 3 days post-silica exposure. We show here that silica-exposed female mice recruit greater numbers of neutrophils and express less SPP1 and greater ICAM-1 mRNA levels compared to male mice at the same time points.

Further studies will assess the role of estrogen in silica-induced neutrophil recruitment, as well as SPP1 and ICAM-1 expression at 3 days post-treatment. Other studies have showed that estrogen regulates neutrophil recruitment (Molloy et al., 2003; Speyer et al., 2005; Y. Wang et al., 2010), SPP1 (Banerjee et al., 2009; Li et al., 2000), and ICAM-1 expression (Tostes, Nigro, Fortes, & Carvalho, 2003). In addition, estrogen will be reduced by ovariectomy in female mice and the effect on neutrophil recruitment studied.

Studies have suggested that neutrophil may play a role in the pathogenesis of pulmonary fibrosis (Crestani et al., 2002; Obayashi et al., 1997). Therefore, assessing the effect of early neutrophil depletion on silica-induced pulmonary fibrosis using an antibody against ICAM-1 can strengthen the results of this study. An earlier study demonstrated that intraperitoneal administration of an ICAM-1 antibody increased neutrophil influx and lung ICAM-1 levels but did not affect lung collagen deposition at 14 days post-silica exposure in mice, implying the partial role of ICAM-1 in this process (Nario & Hubbard, 1996). It was further suggested by the same group that increasing the amount of ICAM-1 antibody and changing the route of administration (intravenous/intra tracheal) may

increase the amount and availability of the antibody. Finally, blocking other molecules associated with neutrophil recruitment such as PECAM-1 and P-selectin may have an effect on collagen deposition (Nario & Hubbard, 1996). Therefore, maximum depletion of neutrophils can be achieved by ensuring time-specific and sustained inhibition within 3 days post-silica exposure.

In conclusion, we show that silica-exposed female mice recruit greater numbers of neutrophils and have a greater degree of lung injury compared to exposed males at 3 days post-treatment. Furthermore, we show that silica-exposed female mice express greater levels of ICAM-1 and less SPP1 compared to exposed males at the same time point. However, SPP1 deficiency upregulates ICAM-1 mRNA levels but does not influence neutrophil recruitment and extent of lung injury at 3 days post-silica exposure. Therefore, SPP1-mediated suppression of ICAM-1 mRNA does not account for the reduced neutrophil recruitment into the lungs in male mice compared to female mice at 3 days post-silica exposure.

4.0 OTHER FINDINGS: SPP1-ITGA9 INTERACTION DOES NOT INFLUENCE GENDER-SPECIFIC SILICA-INDUCED EARLY NEUTROPHIL RECRUITMENT IN MICE

4.1 BACKGROUND

Secreted phosphoprotein 1 when cleaved by thrombin exposes the SVVYGLR (SLAYGLR in mice) motif that binds integrin alpha 4-beta 1(ITGA4-B1) and integrin alpha 9-beta 1 (ITGA9-B1) to mediate its biological activities (Barry et al., 2000; Uede, 2011; Yokosaki et al., 1999). For example SPP1 mediates cell adhesion by binding ITGA4-B1 and SPP1/ITGA4-B1 and SPP1/ITGA9-B1 interactions are associated with migration of leukocytes (Barry et al., 2000; Taooka et al., 1999; Uede, 2011) and survival of neutrophils in rheumatoid arthritis (Sharif et al., 2009). T-cells and macrophages express ITGA4-B1 integrin, while fibroblasts, neutrophils, macrophages, smooth muscle cells and osteoblasts express ITGA9-B1 integrin (Uede, 2011). Intact SPP1 and thrombin cleaved SPP1 are recognized by ITGA4-B1 integrin while only the cleaved form of SPP1 binds to ITGA9-B1 integrin (Uede, 2011).

Interestingly, estradiol and tamoxifen downregulate the expression of the ITGB1 in squamous carcinoma cells (Nelson, Helmstaedter, Moreau, & Lage, 2008), indicating that estrogen may play a role in regulating the ITGA4-B1 and ITGA9-B1 integrins. ITGA9 is important in wound healing, cell adhesion, and migration of fibroblasts (Lenga et al., 2008). ITGA9-B1 and ITGA4-B1 binding of the SLAYGLR sequence of SPP1 may be responsible for hepatic neutrophil infiltration in a rat alcoholic steatohepatitis model (Banerjee et al., 2008). There are suggestions that ITGA9 might induce epithelial to mesenchymal transition by increasing alpha smooth muscle actin (αSMA) expression in

SW480 cells (Gupta, Oommen, Aubry, Williams, & Vlahakis, 2013), which is required in the activation of fibroblasts to myofibroblasts (Banerjee et al., 2008; Kohan, Breuer, & Berkman, 2009b; Pereira et al., 2006). Alpha-SMA is important in wound healing, cell adhesion and migration of fibroblasts, (Lenga et al., 2008) and promotes the survival of synovial fibroblast and NIH cells (Nakayama et al., 2010). A recent study revealed that SPP1 does not induce αSMA in human lung fibroblasts (Pardo et al., 2005). In this study, we hypothesize that female mice recruit greater numbers of neutrophils at pre-fibrotic time points and that an SPP1/ITGA9-B1 interaction mediates neutrophil recruitment to the lungs following exposure to crystalline silica. We show here that gender but not SPP1/ITGA9-B1 interactions influence neutrophil recruitment at pre-fibrotic time points following silica exposure in mice.

4.2 RESULTS

Silica-exposed female mice express less integrin alpha 9 (ITGA9) mRNA and protein compared to male mice at 3 days post-exposure.

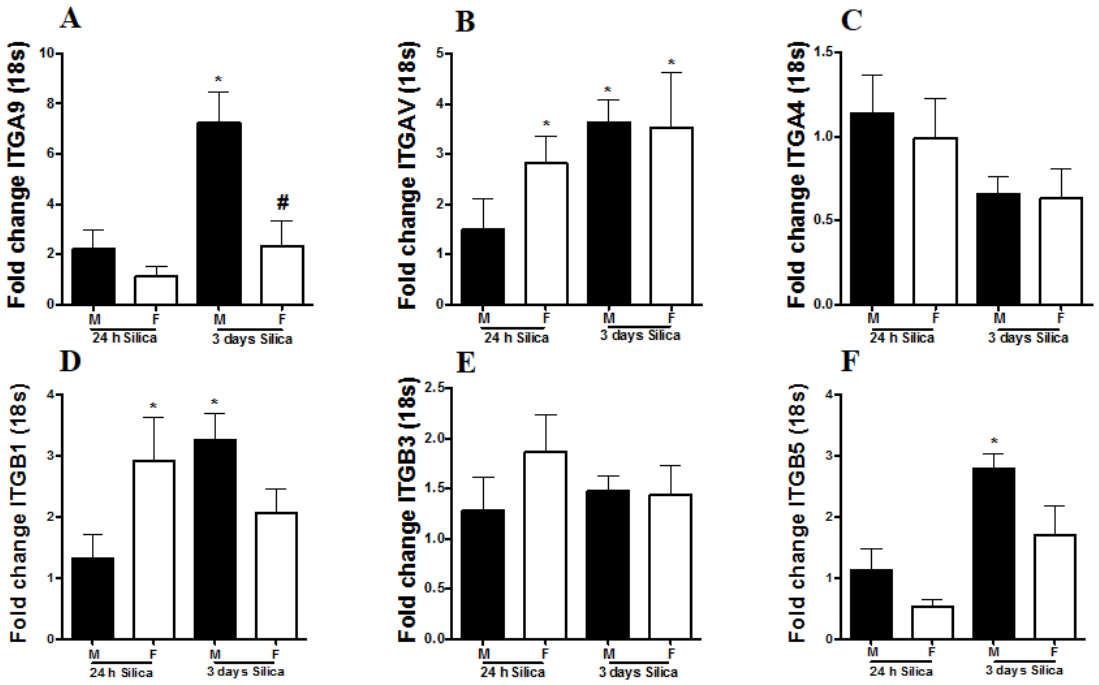
Numerous studies have shown that ITGA9-B1 is associated with neutrophil recruitment to sites of tissue injury and inflammation (Banerjee et al., 2008; Lund et al., 2009; Uede, 2011). To determine the effect of gender on integrin mRNA expression, we assessed the relative mRNA levels of several integrins that interact with SPP1 namely: ITGA9, ITGAV, ITGA4, ITGB1, ITGB3, and ITGB5 by RT-PCR at 24 h and 3 days post-silica exposure.

Figure 15 shows that there is not a significant difference in the mRNA expression levels of ITGA9, ITGAV, ITGA4, ITGB1, ITGB3, or ITGB5 between silica-exposed female and male mice at 24 h post-exposure (Fig: 15 A, B, C, D, E, and F). However, at 3 days

post-treatment, silica-exposed female mice express significantly less ITGA9 mRNA compared to male mice, but no differences were observed between the genders in the expression of the other alpha and beta integrins listed above (Fig: 15 A, B, C, D, E, and F).

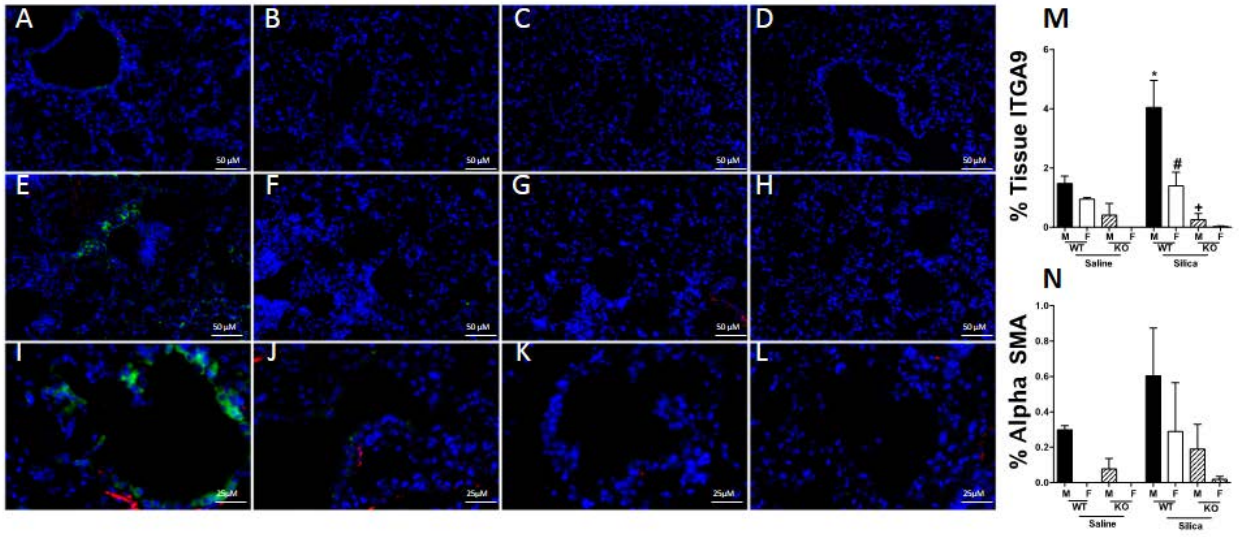
To determine the effect of gender on integrin ITGA9 protein expression, following the observed integrin ITGA9 mRNA expression as shown above, we assessed integrin ITGA9 protein by immunofluorescence staining at 3 days post-silica exposure.

Figure 16 shows that silica-exposed male mice but not female mice express significantly greater amounts of integrin ITGA9 protein compared to the respective saline-exposed controls (Fig: 16 E vs. A, F vs. B, and M). Furthermore, silica-exposed female mice express less integrin ITGA9 protein compared to male mice 3 days post-exposure (Fig: 16 F vs. E, J vs. I, and M).



This panel shows the mRNA expression levels for (A) ITGA9, (B) ITGAV, (C) ITGA4, (D) ITGB1, (E) ITGB3, and (F) ITGB5 at 24 h and 3 days post-silica exposure**.** N ≥ 3per group. * indicates $p<0.05$ vs. same-gender saline, # indicates $p<0.05$ vs. silica-exposed male mice by one-way ANOVA followed by Bonferroni correction for multiple comparisons. NB: mRNA levels in silica-exposed mice were normalized to the respective saline-exposed controls and against Rn18s as an endogenous control (data not shown).

Figure 15: Integrin alpha 9 (ITGA9) mRNA expression is reduced in silica-exposed female mice compared to exposed male mice at pre-fibrotic time points.



The upper panel shows the results of immunofluorescence staining (IMF) for ITGA9 (green) and αSMA (red) in lung tissue from (A) saline-exposed WT male, (B) saline-exposed WT female, (C) saline-exposed SPP1-deficient male, (D) saline-exposed SPP1-deficient female mice at 3 days post-exposure (20X magnification). The middle panel shows IMF staining for ITGA9 and αSMA in lung tissue from (E) silica-exposed WT male, (F) silica-exposed WT female (G) silica-exposed SPP1-deficient male, (H) silica-exposed SPP1-deficient female mice at 3 days post-exposure (20X magnification). The bottom panel shows IMF staining for ITGA9 and αSMA in lung tissue from (I) silica-exposed WT male, (J) silica-exposed WT female (K) silica-exposed SPP1-deficient male, (L) silica-exposed SPP1-deficient female mice at 3 days post-silica exposure (40X magnification)**.** The percentage of tissue staining for ITGA9 at 3 days post-silica exposure is shown in (M). The percentage of tissue staining for αSMA at 3 days post-silica exposure is shown in (N). N ≥ 3 per group, * indicates $p<0.05$ vs. same-gender saline, # indicates $p<0.05$ vs. silica-

exposed male mice, + indicates p<0.05 vs. wild type mice by one-way ANOVA followed by Bonferroni correction for multiple comparisons.

Figure 16: Silica-exposed female mice express less integrin alpha 9 (ITGA9) protein compared to male mice. SPP1 plays a role in ITGA9 expression in male mice but not female mice at 3 days post-silica exposure.

SPP1 plays a role in ITGA9 expression in silica-exposed male mice but not female mice and has no effect on other integrins and αSMA at 3 days post-exposure.

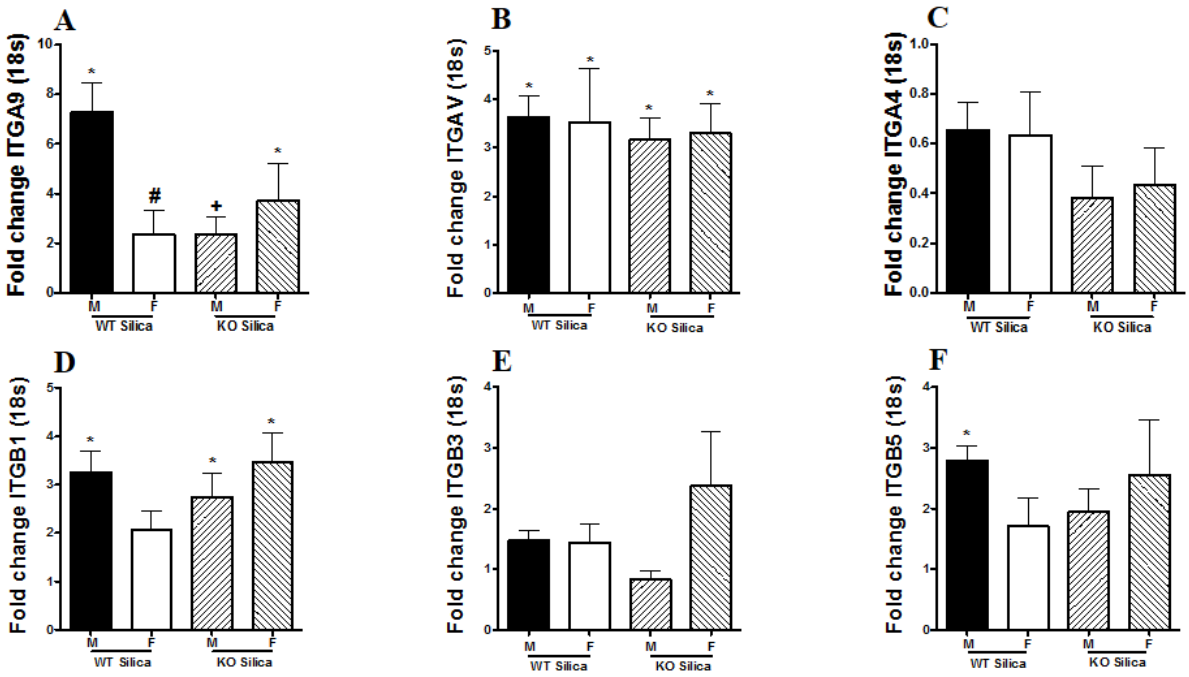
Studies have demonstrated that SPP1 is required for the activation of fibroblasts to myofibroblasts (Kohan et al., 2009b; Lenga et al., 2008; Pereira et al., 2006). ITGA9 has also been shown to be important in would healing, the adhesion and migration of fibroblasts (Nakayama et al., 2010) and inhibiting anoikis in synovial fibroblasts and NIH cells (Kanayama et al., 2009).

To determine the effect of SPP1 on integrin and αSMA expression, we exposed male and female wild type and SPP1-deficient mice to silica and assessed the relative mRNA expression of ITGA9, ITGAV, ITGA4, ITGB1, ITGB3, ITGB5 by RT-PCR and ITGA9 and αSMA protein by immunofluorescence staining at 3 days post-exposure.

Figure 17 shows that silica-exposed SPP1-deficient male mice but not females express significantly less ITGA9 mRNA compared to the respective gender wild type mice (Fig: 17A). Furthermore, the mRNA expression of the other integrins is not significantly different between silica-exposed SPP1-deficient mice and the respective gender wild type mice (Fig: 17 B, C, D, E and F). There is not a significant difference in the mRNA expression of any of the integrins between the SPP1-deficient female and male mice at 3 days post-silica exposure (Fig: 17 A, B, C, D, E and F). Similarly, there is not a significant

difference in ITGA9 protein expression between SPP1-deficient female and male mice at 3 days post-exposure (Fig: 16 H vs. G, L vs. K and M). However, silica-exposed SPP1-deficient male mice but not females express significantly less ITGA9 protein compared to the respective gender wild type mice (Fig: 16 G vs. E, H vs. F, K vs. I, L vs. J, and M). In addition, neither the αSMA expression between silica-exposed mice (wild type and SPP1-deficient) and the respective saline-exposed controls (Fig: 16 E vs. A, F vs. B, G vs. C, H vs. D, and N), nor between silica-exposed SPP1-deficient mice and the respective exposed wild type mice (Fig: 16 G vs. E, H vs. F, K vs. I, L vs. J, and N) is significantly different.

Finally, there is not a significant difference in αSMA expression between silica-exposed female and male mice at 3 days post-exposure (Fig: 16 F vs. E, H vs. G, J vs. I, L vs. K, and N).



This panel shows the mRNA expression levels for (A) ITGA9, (B) ITGAV, (C) ITGA4, (D) ITGB1, (E) ITGB3, and (F) ITGB5 in silica-exposed wild type (WT) and SPP1-deficient male and female mice at 3 days post-silica exposure. N ≥ 3 per group, * indicates $p<0.05$ vs. same-gender saline, # indicates $p<0.05$ vs. silica exposed male mice, + indicates $p<0.05$ vs. wild type mice by one-way ANOVA followed by Bonferroni correction for multiple comparisons. NB: mRNA levels in silica-exposed mice were normalized to the respective saline-exposed controls and to Rn18s as an endogenous control (data not shown).

Figure 17: SPP1 influences ITGA9 mRNA expression in silica-exposed male mice but not female mice at 3 days post-exposure.

4.3 DISCUSSION

Numerous studies have shown that integrin alpha 9-beta 1(ITGA9-B1) is associated with neutrophil recruitment to sites of tissue injury and inflammation (Denhardt, Giachelli, et al., 2001; Nau et al., 1997; Uede, 2011) and mediates cell adhesion (Smith et al., 1996; Yokosaki et al., 1999). ITGA9-B1 and ITGA4-B1 binding of the SLAYGLR sequence of SPP1 may be responsible for hepatic neutrophil infiltration in a rat alcoholic steatohepatitis model (Banerjee et al., 2008). ITGA9 is expressed in the airway epithelium, the basal layer of squamous epithelium, smooth and skeletal muscle and hepatocytes (Palmer, Ruegg, Ferrando, Pytela, & Sheppard, 1993). We showed that female mice express less ITGA9 mRNA and protein compared to male mice at 3 days post-silica exposure (Fig: 15A, Fig: 16 F vs. E, J vs. I, and M). This is contrary to the earlier observation showing greater numbers of recruited neutrophils in female mice compared to male mice (Fig: 8F). We also observed that SPP1-deficiency decreases ITGA9 mRNA and protein expression in male mice but does not have a significant effect on female mice at 3 days post silica exposure (Fig: 17A, Fig: 16 G vs. E, H vs. F, K vs. I, L vs. J, and M). We also showed that gender and SPP1 do not affect the mRNA expression levels of all the other integrins studied (ITGAV, ITGA4, ITGB1, ITGB3 and ITGB5- Fig: 17 B, C, D, E and F). This indicates that SPP1-ITGA9 interactions might not be the major pathway involved in determining the gender-specific differences in neutrophil recruitment at pre-fibrotic time points. There are suggestions that ITGA9 might induce epithelial to mesenchymal transition by increasing alpha smooth muscle actin (αSMA) expression in SW480 cells (Gupta et al., 2013) Alpha-SMA is required in the activation of fibroblasts to myofibroblasts (Banerjee et al., 2008; Kohan et al., 2009b; Pereira et al.,

2006) and it is important in wound healing, the cellular adhesion and migration of fibroblasts (Lenga et al., 2008) and promoting the survival of synovial fibroblasts and NIH cells (Nakayama et al., 2010). A recent study revealed that SPP1 does not induce αSMA in human lung fibroblasts (Pardo et al., 2005). We showed that there is not a significant difference in the expression of αSMA protein between female and male mice at 3 days post silica exposure (Fig: 16 F vs. E, H vs. G, J vs. I, L vs. K, and N). Furthermore, SPP1 deficiency did not affect the expression of αSMA at 3 days post-silica exposure (Fig: 16 G vs. E, H vs. F, K vs. I, L vs. J, and N). We also showed that ITGA9 and αSMA colocalize in airway epithelium (Fig: 16 E and I). This observation indicates that 3 days post-silica exposure might be too early to observe the activation of fibroblasts.

In conclusion, we show that gender but not SPP1/ITGA9 interactions influence silica-induced neutrophil recruitment and tissue injury at pre-fibrotic time points.

5.0 CONCLUSION

The overall aim of this dissertation is to evaluate the role of estrogen in silica-induced pulmonary fibrosis and the roles of gender and SPP1 in silica-induced early neutrophil recruitment in mice.

Estrogen protects against silica-induced pulmonary fibrosis through the downregulation of SPP1.

We previously showed that silica-exposed female mice have less severe pulmonary fibrosis compared to exposed males, while ovariectomized mice show fibrosis similar to males, at 14 days post-exposure (Brass et al., 2010). Consistent with the above findings, we show here that estrogen-treated male mice recruit greater numbers of inflammatory cells that were predominantly macrophages compared to vehicle-treated male mice at 14 days post-silica exposure (Fig: 3 A and B). This is similar to the inflammatory response seen in silica-exposed female mice. In addition, estrogen-treated male mice have decreased collagen deposition and fibrosis compared to vehicle-treated male mice at 14 days post-silica treatment (Fig: 4 D vs. C, E, and Fig: 5). Likewise, this is similar to the pattern of collagen deposition and fibrosis observed in female mice in response to silica. Therefore, estrogen supplementation of male mice causes the inflammatory response and extent of fibrosis to be similar to that of female mice, suggesting that estrogen plays a role in silica-induced pulmonary fibrosis in mice.

Earlier studies show that SPP1 is important in animal models of pulmonary fibrosis and IPF. For example, SPP1 expression is upregulated in the lung after exposure to chrysolite asbestos (Sabo-Attwood et al., 2011), and bleomycin (Takahashi et al., 2001b),

while a decrease in asbestos-induced injury and inflammation was observed in SPP1-deficient mice (Sabo-Attwood et al., 2011). SPP1 is elevated in the lungs of IPF patients relative to normal lungs and increased in the BAL of IPF patients (Pardo et al., 2005). We previously showed that SPP1 levels are elevated in silica-exposed mice compared saline-treated control mice. We also showed that silica-exposed female mice express less SPP1 compared to exposed male, and that SPP1-null mice showed reduced silica-induced pulmonary fibrosis (unpublished data). Furthermore, estrogen regulation of SPP1 depends upon the cellular context. For example, estrogen inhibits SPP1 expression in rat vascular smooth muscle cells (Li et al., 2000) and ovariectomy suppresses the expression of SPP1 in the rat kidney (Miyajima et al., 2010). Interestingly, estrogen-treated male mice have reduced SPP1 expression compared to vehicle treated male mice in response to silica (Fig: 6 D vs. C, E and F). Once more, this is similar to pattern of SPP1 expression seen in female mice in response to silica, suggesting that estrogen regulate silica-induced pulmonary fibrosis through the downregulation of the profibrotic cytokine, SPP1.

Gender plays a role in the early neutrophil recruitment after silica in mice.

As mentioned above, at 14 days post-silica exposure, macrophages are the predominant inflammatory cell recruited into the lungs (Brass et al., 2010; Ramsgaard et al., 2010). However, at earlier time points following silica exposure, neutrophils are the predominant inflammatory cells (Nario & Hubbard, 1997; Sato et al., 2008). Other groups have also observed a gender bias in immune cell recruitment. It is known that estrogen also increases neutrophil survival (Molloy et al., 2003) and significantly increases lung inflammation and neutrophil recruitment in certain circumstances (Y. Wang et al., 2010) but not others (Speyer et al., 2005). We show in this study that female mice recruit greater

numbers of neutrophils and have greater extent of lung injury compared to male mice at 3 days post-silica exposure (Fig: 8F, Fig: 9 H vs. G and K). Numerous studies have indicated that ICAM-1 is critical in neutrophil recruitment to sites of injury (Basit et al., 2006; Nario & Hubbard, 1996; Ochietti et al., 2002; Pang et al., 2008; L. Yang et al., 2005) and plays a role in silica-induced neutrophil recruitment in mice (Hubbard et al., 2001; Nario & Hubbard, 1996). Estrogen increases the expression of tumor necrosis factor-induced ICAM-1, but has an opposite effect at 6 h post-treatment (Cid et al., 1994). We show in this study that silica-exposed female mice express higher levels of ICAM-1 mRNA compared to exposed males at both 24 h and 3 days post-treatment (Fig: 10A). Therefore, we suggest that ICAM-1 may play a role in the gender-specific neutrophil recruitment at pre-fibrotic time points.

Loss of SPP1 does not influence early neutrophil recruitment after silica exposure in mice.

SPP1 is chemotactic to neutrophils (van der Windt et al., 2010) but SPP1 deficiency does not affect the phagocytic ability or the generation of ROS by neutrophils (Koh et al., 2007). In addition, SPP1 deficiency does not influence neutrophil recruitment in response to Streptococcus pneumonia (van der Windt et al., 2011). Silica-exposed female mice express less SPP1 compared to exposed males at pre-fibrotic time points (Fig: 11 D vs. C, H vs. G, I, J, and K). In addition, loss of SPP1 abolishes the gender-specific difference in neutrophil recruitment and lung injury at 3 days post-silica exposure (Fig: 12B, Fig: 13 H vs. G and I). However, SPP1 did not influence neutrophil recruitment in same gender mice (Fig: 12B). Furthermore, SPP1 deficiency downregulates ICAM-1 at 3 days post-silica exposure (Fig: 14 A and B). Taken together, the fact that silica-

exposed female mice recruit greater numbers of neutrophils and have higher ICAM-1 levels, we conclude that gender but not SPP1 influences neutrophil recruitment at pre-fibrotic times after silica exposure.

Future directions

A previous study showed that estradiol injection into ovariectomized rat diminished bleomycin-induced pulmonary fibrosis (Carey, Card, et al., 2007a). Further studies will assess the effect of estrogen in silica-induced pulmonary fibrosis in ovariectomized mice. Since ovariectomized mice have silica-induced pulmonary fibrosis similar to males (Brass et al., 2010), we expect estradiol to reverse the increase in pulmonary fibrosis. We previously showed that estrogen receptor alpha (ERα) but not estrogen receptor beta (ERβ) is differentially expressed based on gender with male mice having higher levels of ERα compared to female mice at 14 days post-silica treatment (Brass et al., 2010). It has been established that viral-vector mediated siRNA can be delivered locally to silence ERα expression (Ribeiro et al., 2012) and tissue specific knockdown of ERα by Cre-LoxP technology is available through Jackson Laboratory. In view of this, we can further strengthen this study by assessing the effect of conditional knock down of ERα in the lungs in silica-induced pulmonary fibrosis in mice.

Since SPP1 does not account for the gender-specific difference in neutrophil recruitment, there is need to explore other mechanism to explain this observed phenomenon. Studies have showed that estrogen regulates neutrophil recruitment (Molloy et al., 2003; Speyer et al., 2005; Y. Wang et al., 2010), SPP1 (Banerjee et al., 2009; Li et al., 2000), and ICAM-1 expression (Tostes et al., 2003). To demonstrate

the role of estrogen in early neutrophil recruitment, male and female mice will be pre-treated with estrogen for three weeks then treated with silica for three days. We will then assess inflammation in the BAL fluid by Hema Protocol 3 stained differential inflammatory cell count, lung tissue injury by Mason's trichrome staining and finally ICAM-1 mRNA and protein by RT-PCR and immunohistochemistry and western blot respectively. In addition, female mice will be ovariectomized to reduce estrogen and the effect of this reduction on neutrophil recruitment will be assessed as in above. Studies have suggested that neutrophil may play a role in the pathogenesis of pulmonary fibrosis (Crestani et al., 2002; Obayashi et al., 1997). Assessing the effect of early neutrophil depletion on silica-induced pulmonary fibrosis using an antibody against ICAM-1 can strengthen the results of this study. An earlier study demonstrated that intraperitoneal administration of an ICAM-1 antibody decreased neutrophil influx and lung ICAM-1 levels but did not affect lung collagen deposition at 14 days post-silica exposure in mice, implying the partial role of ICAM-1 in this process (Nario & Hubbard, 1996). It was further suggested by the same group that increasing the amount of ICAM-1 antibody and changing the route of administration (intravenous/intra tracheal) may increase the amount and availability of the ICAM-1 antibody. Finally, blocking other molecules associated with neutrophil recruitment like PECAM-1 and P-selectin may have an effect on collagen deposition (Nario & Hubbard, 1996). Therefore, maximum depletion of neutrophils can be achieved by ensuring time-specific and sustained inhibition within 3 days post-silica exposure.

The overall goal of this dissertation was to investigate the role of estrogen in silica-induced pulmonary fibrosis and the roles of gender and SPP1 in silica-induced early neutrophil in mice. Finally, we have shown that:

1) Estrogen supplementation of male mice is protective against silica-induced pulmonary fibrosis through downregulation of the pro-fibrotic cytokine SPP1.
2) SPP1 modulation of ICAM-1 does not influence the gender-specific differences in early neutrophil recruitment to the lungs post-silica exposure.

From a public health perspective, this study will broaden the knowledge of silicosis and provides further insight into preventive and therapeutic measures to slow the progression of or cure the disease. In addition, the study may help identify candidate genes and properly advise susceptible individuals or workers against exposure to silica. This research effort may help identify biomarkers for early diagnosis and timely management of silicosis. Finally, successful research will lead to overall reduction in the global burden of silicosis and promotion of public health.

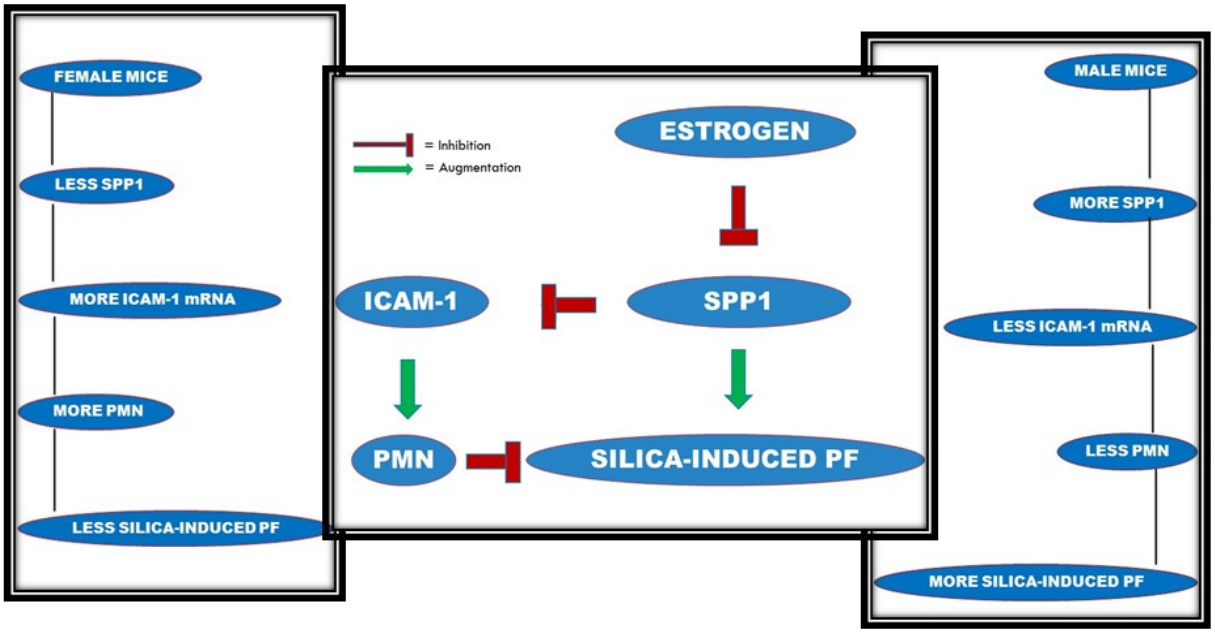


Figure 18: Schematic representation of the molecular mechanism for the gender-specific differences in silica-induced PF and early neutrophil recruitment in mice.

6.0 MATERIALS AND METHODS

6.1 MATERIALS

Male and female C57BL/6J and SPP1 (-/-) (B6.Cg-SPP1^{tm1Blh}/J) mice were obtained from Jackson Laboratories (Bar Harbor, ME). Min-U-Sil 5 was graciously provided by Dr. Andy Ghio (Environmental Protection Agency, Durham, NC). Kethasthesia was purchased from Butler-Schein (Dublin Ohio). Xylazine hydrochloride was obtained from MP Biomedicals (Solon, OH). The SPP1 ELISA and antibody were purchased from R&D systems (Minneapolis, MN). Immunohistochemistry was performed using a secondary antibody and Vectastain Elite ABC kit from Vector Laboratories (Burlington, CA) and an amino-ethylcarbazole substrate kit from Life Technologies (Camarillo, CA). Immunofluorescence staining was performed using Alexa 488 and 568 secondary antibodies and ProLong Gold antifade reagent with DAPI from Invitrogen Eugene, Oregon). Mason's trichrome reagents and hematoxylin were purchased from Sigma-Aldrich (St Louis, MO). All other reagents were purchased from Fisher Scientific (Pittsburgh, PA).

6.2 ANIMAL TREATMENT AND INTRATRACHEAL INSTILLATION

Part 1:

We divided 8-10 weeks old male C57BL/6J mice into two groups, pre-treated with subcutaneous injections of 250 ng of 17-β estradiol (Sigma-Aldrich-E8875, St Louis, MO) or an equivalent volume of olive oil (Sigma-Aldrich-O1514) as a vehicle control for 21 days. The mice were then transiently anaesthetized with inhaled isoflurane and given a single intratracheal dose of 0.2 mg/kg of Min-U-Sil-5 freshly fractured crystalline silica or 0.9% saline in a total volume of 60 µl. Mice were sacrificed at 14 days post-exposure.

Part 2:

Male and female C57BL/6J and SPP1 (-/-) mice were transiently anaesthetized with inhaled isoflurane and given a single intratracheal dose of 0.2 mg/kg of Min-U-Sil 5 freshly fractured crystalline silica or 0.9% saline in a total volume of 60 µl. Mice were sacrificed at 24 h or 3 days post-exposure. All animal procedures were performed in accordance with University of Pittsburgh Institutional Animal Care and Use Committee approved protocols.

6.3 SAMPLE PROCESSING

Saline- and silica-treated mice were euthanized at 24 h, 3 days or 14 days post-exposure using 20 mg/kg ketamine and 2 mg/kg xylazine. BAL fluid was obtained by instilling and withdrawing 1300 µl of sterile 0.9% saline. The right lungs were flash frozen in liquid nitrogen and stored at -80 °C, while the left lungs were fixed in 10% buffered formalin (gravity flow at 10 mmHg) and processed for routine histological analysis.

6.4 LAVAGE TOTAL CELL COUNT AND DIFFERENTIAL CELL COUNT

Total leukocyte cell counts in BAL fluid were determined using a Beckman Dual Z1 Coulter particle counter (Coulter, Fullerton, CA). White blood cell differential counts were obtained by staining cytospins of BAL fluid with Protocol Hema 3 stain set (Fisher Diagnostics, Middletown, VA). The percentage and numbers of total cells that were macrophages, lymphocytes and neutrophils were determined by light microscopy.

6.5 SPP1 ANALYSIS IN BAL FLUID

The enzyme-linked immunosorbent assay (ELISA) for SPP1 was performed using the SPP1 ELISA DuoSet according to manufacturer’s instructions. The absorbance at 450 nM was read using a SpectraMax M2e plate reader (Molecular Dynamics, Sunnyvale,

California) and standard curves and sample values were generated using the SoftMax Pro software.

6.6 HISTOCHEMICAL ANALYSIS OF FORMALIN-FIXED LUNG TISSUE

6.6.1 Trichrome staining

Lung sections were deparaffinized, rehydrated and stained with Masson's trichrome according to manufacturer's instructions (HT15 Sigma-Aldrich, St Louis, MO). Photomicrographs were captured using a Nikon Eclipse 90i microscope.

NB: Percent tissue volume = Percentage of lung tissue that are cells + Percentage of lung tissue that is collagen. This is done by color threshold and pixel analysis using the Nikon Elements analysis software for each photomicrograph. Ten representative images are taken for each lung section and average percent tissue volume is calculated for each mouse. NB: Red = cells; Blue = collagen; White = air space.

6.6.2 Immunohistochemistry Staining

Tissue sections for immunohistochemical analysis were deparaffinized and rehydrated. Antigen was retrieved by three microwave exposures (20% power for 5 minutes) in citrate buffer (1mM, pH 6.0) and then endogenous peroxidases quenched by incubation with 1% hydrogen peroxide for 10 minutes. The sections were blocked with 5% horse serum for 45 minutes, then a goat polyclonal antibody to mouse SPP1 was applied and the samples incubated for 1 h at room temperature (2 µg/mL, 1:100 dilution, AF-808, R&D, Minneapolis, MN).

Samples were then incubated in a biotinylated horse anti-goat secondary for 45 minutes at room temperature (1:200 dilution, BA-9500, Vector laboratories Inc., Burlingame, CA). This was followed by incubation with the Vectastain Elite ABC reagent

for 30 minutes according to manufacturer's instructions (PK-6100, Vector laboratories Inc., Burlingame, CA). AEC (red) was used as the substrate according to manufacturer's instructions (00-2007, Invitrogen Corporation, Camarillo, CA). Finally, the sections were counterstained with hematoxylin. The primary antibody was replaced with 4% BSA in 1X PBS for the negative control slides. Lung SPP1 was quantified by color thresholding analysis using Nikon Elements analysis software as previously described (Brass et al., 2010). NB: Red = SPP1; Pink = nuclei; White = air space.

6.6.3 Immunofluorescence staining

Tissue sections for immunofluorescence analysis were deparaffinized and rehydrated. Antigen was retrieved as above and then the membrane was permeabilized by incubating in 0.1% Triton X100 for 15 minutes. Sections were blocked with 20% donkey serum for 45 min at room temperature, then coincubated with rabbit monoclonal antibody to integrin alpha 9 (ITGA9) (1:50 dilution, ab140599, Abcam, Cambridge, MA), and a goat polyclonal antibody to alpha smooth muscle actin (αSMA) 1:75 ab21027, Abcam) overnight at 4 °C Sections were then coincubated with an Alexa 488 donkey anti-rabbit and an Alexa 568 donkey anti-goat secondary antibody for one h (Life Technologies, City, State). This was followed by mounting the sample with the ProLong gold anti fading reagent with DAPI (Life Technologies, Eugene, Oregon). The primary antibody was replaced with 0.5% BSA in 1X PBS for the negative control. Lung ITGA9 was quantified by color thresholding analysis using the Nikon Element analysis software, as noted above. NB: Green = ITGA9; Blue = nuclei; Red = αSMA Black = air space.

6.7 RT-QPCR ANALYSIS

Lung tissue was homogenized and total RNA isolated using TRIZOL (Sigma-Aldrich, St Louis, MO) according to manufacturer's instructions. The isolated RNA was purified using a DNA-free kit per the manufacturer's instructions (AM1906, Life Technologies, Carlsbad, CA) and quantified by A260/A280 spectrophotometric absorbance using a BioTek Synergy 2 multimode microplate reader and the Gen5 data analysis software. For real time quantitative PCR (RT-QPCR) 200 ng of RNA was reversed transcribed using an iScript CDNA synthesis kit (1708891, BioRad, Hercules, CA) for 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C. One microliter of cDNA was used for each 10 µl qRT-PCR reaction consisting of 5 µl TaqMan Gene Expression master mix, 3.5 µl of ultrapure water, and 0.5 µl of SPP1 (Mn01611440_mH), ICAM-1 (Mm00516023_m1), MIP-2 (Mm00436450_m1), VEFG-A (Mm01281449_m1), ITGA9 (Mm01348483_m1), ITGAV (Mm00434486_m1), ITGA4 (Mm01277951_m1), ITGB1 (Mm01253230_m1), ITGB3 (Mm00443980_m1), ITGB5 (Mm00439825_m1) and Rn18s (Mm03928990_g1) as endogenous control (Applied Biosystems-Life Technologies, Grand Island, NY, City, State). The reaction cycle using ABI 7900HT fast Real Time PCR System is a follows: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 sec at 95 °C followed by 1 min at 60 °C. The relative levels of SPP1 transcripts was analyzed and expressed as $\Delta\Delta C_T$.

6.8 LUNG COLLAGEN CONTENT ANALYSIS

The lungs were placed individually into 2 ml glass ampules and dried at 110 °C. After drying, 2 ml of 6 N HCl was added to each ampule. Oxygen was purged from each ampule and replaced with nitrogen, the ampules were vacuum-sealed and the samples

acid hydrolyzed at 110 °C for 24 h. The acid was completely driven off by incubating at 110 °C prior to suspension in 2 ml of 1X PBS at 60 °C for 1 h.

The rehydrated samples were centrifuged at 13,000 rpm for 10 min to remove particulate matter. The hydroxyproline content of the resulting supernatants was determined using chloramine T as previously described (Fattman, Chu, Kulich, Enghild, & Oury, 2001; Woessner, 1961).

6.9 STATISTICAL ANALYSIS

All data are expressed as means ± SEM and evaluated by one-way ANOVA using Bonferroni's post-hoc test for multiple comparisons. Data with P values ≤ 0.05 were considered significant using PRISM (Graph pad, version 5.0b). All animal groups had an N ≥ 3.

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