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## **Secreted Phosphoprotein 1 and Sex-Specific Differences in Silica-Induced Pulmonary Fibrosis in Mice**

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**Running title:** SPP1 and sex-specific responses to silica in mice

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## Abstract

**Background:** Fibrotic lung diseases occur predominantly in males and reports describe better survival in affected females. Male mice are more sensitive to silica-induced lung fibrosis compared to silica-treated female mice. Secreted phosphoprotein 1 (SPP1, aka osteopontin) increases in pulmonary fibrosis, and SPP1 transcription may be regulated by estrogen or estrogen receptor-related receptors.

**Objective:** To determine whether differences in silica-induced SPP1 expression contributes to sex differences in lung fibrosis.

**Methods:** Male and female mice were treated with 0.2g/kg intratracheal silica and lung injury was assessed 1, 3, or 14 days post-exposure. Gene-targeted (*Spp1*<sup>-/-</sup>) mice, control *Spp1*<sup>+/+</sup> (C57BL/6J) mice, ovariectomized (OVX) female mice, or estrogen-treated male mice were treated with silica and lung injury was assessed.

**Results:** Silica-induced SPP1 in lung tissue, bronchoalveolar lavage, and serum increased more in male than female mice. Following silica treatment, bronchoalveolar lavage cell infiltrates decreased in female *Spp1*<sup>-/-</sup> mice compared to female *Spp1*<sup>+/+</sup> mice, and lung hydroxyproline decreased in male *Spp1*<sup>-/-</sup> mice compared to male *Spp1*<sup>+/+</sup> mice. OVX female mice had increased lung SPP1 expression in response to silica compared to silica-treated sham female mice. Silica-induced lung collagen and hydroxyproline (markers of fibrosis), and SPP1 expression decreased in estrogen treated males compared to untreated males.

**Conclusion:** These findings suggest that sex-specific differences in SPP1 expression contribute to the differential sensitivity of male and female mice to the development of silica-induced fibrosis.

## Introduction

A chronic fibrotic lung disease caused by silica inhalation, silicosis is a detrimental occupational disease, with thousands of new cases being reported worldwide annually (Leung et al. 2012). Hazardous occupational exposures occur in mining, sandblasting, road construction, pottery making, masonry, and tunneling operations. Recently, silica exposures have occurred during hydraulic fracturing of gas and oil wells (Esswein et al. 2013), and during fabrication and installation of engineered stone countertops (Kramer et al. 2012). Non-occupational silica exposures can result near industrial or non-industrial sources (Bhagia 2012). In the United States, age-adjusted mortality rates have dropped from 8.9/million in 1968 to 0.4/million in 2010 (Bang et al. 2015). In recent years, silicosis has increased with increased coal workers' pneumoconiosis (Halldin et al. 2015; Laney and Weissman 2014) and patients are younger and develop accelerated, severe silicosis with higher mortality (Laney and Weissman 2014).

Most silicosis patients are men as occupations associated with silicosis were historically male-dominated (Leung et al. 2012) and limited data suggest that survival may be better in women (Morozova 2012). Similarly, other fibrotic lung diseases (e.g., idiopathic pulmonary fibrosis) also occur predominantly in men (Raghu et al. 2006; Hutchinson et al. 2014) and women experience better survival (Schwartz et al. 1994; McCormick et al. 1995; Gribbin et al. 2006; Han et al. 2008; Raghu et al. 2014). This suggests that sex can influence pulmonary fibrosis pathogenesis. However, how sex alters the development and progression of pulmonary fibrosis is largely unknown, inasmuch as most studies control for sex rather than examine sex-specific effects. Furthermore, animal studies fail to clearly define the relationship between sex and pulmonary fibrosis (Carey et al. 2007). For example, female rats develop more severe bleomycin-induced pulmonary fibrosis than male rats (Gharaee-Kermani et al. 2005), whereas

male mice develop more severe bleomycin-induced pulmonary fibrosis than female mice male (Redente et al. 2011; Voltz et al. 2008).

Secreted phosphoprotein-1 (SPP1) is an extracellular matrix protein and cytokine associated with inflammatory and profibrotic effects in a number of organs (Wang and Denhardt 2008). In the lung, macrophages, lymphocytes, and alveolar epithelial cells produce SPP1 (Ganguly et al. 2014; O'Regan 2003). As a pleiotropic cytokine, SPP1 stimulates macrophage and neutrophil chemotaxis, type-1 cytokine secretion, and macrophage and lymphocyte differentiation (Lund et al. 2009; Wang and Denhardt 2008). As a pro-fibrotic molecule, SPP1 controls expression and augments the effects of other profibrotic mediators, such as transforming growth factor, beta 1 (TGFB1) on fibroblast proliferation and myofibroblastic differentiation (Nagao et al. 2012; Xiao et al. 2012). SPP1 also mediates fibroblast migration through integrin- (Anwar et al. 2012; Li et al. 2000) and matrix metalloproteinase-dependent mechanisms (Lund et al. 2009). Lung SPP1 increases in humans with pulmonary fibrosis (Foster et al. 2015; Nau et al. 1997; Pardo 2005) and in mouse (Berman et al. 2004; Miyazaki Y et al. 1995; Oh et al. 2015; Sabo-Attwood et al. 2011; Takahashi et al 2001) and rat (Langley et al. 2011; Ma 2012; Mangum 2004) models of pulmonary fibrosis. In addition, SPP1 may be a useful biomarker for development and progression of fibrotic lung diseases (Boon et al. 2009; Kadota et al. 2005; Kelly et al. 2006; O'Regan et al. 2006; Pardo et al. 2005; Selman et al. 2006; Vij and Noth 2012). Gene-targeted *Spp1*<sup>-/-</sup> mice developed less bleomycin-induced pulmonary fibrosis (Berman et al. 2004) as well as reduced injury in models of renal, heart, kidney and liver fibrosis (Rittling and Denhardt 1999).

Previously, we observed that silica-treated male mice develop increased fibrosis but a weaker inflammatory response compared to female mice (Brass et al. 2010). Studies of *SPP1* promoter

suggested that estrogen could activate transcription not through classical estrogen response element (ERE) but through estrogen receptor 1 interactions with SF-1 response elements (SFRE) in the promoter (Craig and Denhardt 1991; Vanacker et al. 1999). However, regulation of the *SPP1* promoter is complex and may be tissue- or disease-context dependent. For example, in osteoblasts, *SPP1* also can be activated or repressed by estrogen receptor-related receptors (ESRRs) that also bind SFRE sites in *SPP1* promoter (Zirngibl et al. 2013). Although these orphan receptors do not bind estrogen, ESRRs can interact with estrogen receptors (Johnston et al. 1997), leaving the role of estrogen in regulating *SPP1* uncertain. Thus, this study examines whether altered *SPP1* expression is associated with sex difference observed in silica-induced pulmonary fibrosis in mice.

## Materials and Methods

**Materials:** Male and female *Spp1*<sup>+/+</sup> (C57BL/6J), ovariectomized (OVX) *Spp1*<sup>+/+</sup> (C57BL/6J) female, and *Spp1*<sup>-/-</sup> (B6.Cg-*Spp1*<sup>tm1Blh</sup>/J) (Liaw et al. 1998) mice were obtained from Jackson Laboratories (Bar Harbor, ME). Studies with ovariectomized (OVX) mice were compared to Sham mice that had similar surgery without removal of the ovaries (Jackson Laboratories). Studies were conducted with C57BL/6J male mice injected subcutaneously with 250 ng of 17-beta estradiol or equal volume (50  $\mu$ l) of vehicle (olive oil) daily for 21 days before silica exposure. Silica (Min-U-Sil 5) was provided by Dr. Andy Ghio, (Environmental Protection Agency, Durham, NC). The following chemicals were obtained from identified suppliers: isoflurane (Webster Veterinary, Devens, MA), ketasthesia (Butler-Schein, Dublin, OH), xylazine hydrochloride (MP Biomedicals, Solon, OH), *SPP1* ELISA and antibody (R&D Systems, Minneapolis, MN), and Masson's trichrome reagents and hematoxylin (Sigma Chemicals, St Louis, MO). Immunohistochemistry was performed using secondary antibodies

with Vectastain Elite ABC (Vector Labs, Burlingame, CA) and amino-ethylcarbazole substrate (Life Technologies, Camarillo, CA). All other chemicals and reagents were purchased from Thermo Fisher Scientific (Pittsburgh, PA).

**Intratracheal Instillations:** Animal procedures were approved by University of Pittsburgh Institutional Animal Care and Use Committee and mice used were treated humanely and with regard for alleviation of suffering. Male and female mice were anesthetized briefly with inhaled isoflurane and given a single intratracheal dose of 0.2 mg/kg Min-U-Sil 5 crystalline silica (median aerodynamic diameter = 2.2  $\mu\text{m}$ ) or 0.9% saline in a total volume of 60  $\mu\text{l}$ . Mice were sacrificed 14 days post-exposure.

**Sample Processing:** Saline and silica-treated mice were euthanized at 1, 3 or 14 days post-exposure using 20 mg/kg ketamine and 2 mg/kg xylazine. Bronchoalveolar fluid (BALF) was obtained by instilling and withdrawing 1.3 ml of sterile 0.9% saline. Right lungs were excised and acid hydrolyzed for hydroxyproline determination. Left lungs were either flash-frozen in liquid nitrogen (stored at  $-80^{\circ}\text{C}$ ) or fixed in 10% buffered formalin (gravity flow at 10mm Hg) and processed for routine histological analysis. At day 14, blood samples were collected via intracardial puncture. Serum was obtained by centrifugation at 5000 rpm for 5 minutes at RT and stored at  $-80^{\circ}\text{C}$ .

**Lavage Total Cell Count, Differential Cell Count and Protein Determination:** Total leukocyte cell counts in BALF were determined using a Beckman Dual Z1 Coulter Particle Counter (Coulter, Fullerton, CA). White blood cell differential counts were obtained by staining cytopins of BALF with a Protocol Hema 3 stain (Thermo Fisher Scientific). The percentage and number of total cells and that were macrophages, lymphocytes and neutrophils were determined by light microscopy. Total BALF protein content was determined using Coomassie Plus

colorimetric assay. Sample absorbance was measured at 595 nm using a Beckman Coulter DU800 spectrophotometer.

**Histochemical Analysis of Formalin-fixed lung tissue:** Lung sections were stained with Masson's trichrome and photomicrographs were captured using a Nikon microscope and Nikon Elements analysis software. Lung sections were stained with Masson's trichrome to visualize collagen. The entire lung was photographed at  $\times 20$  magnification, and Nikon Elements software was used to calculate tissue volume density (TVD) defined as the percentage of each microscopic field that is lung tissue as well as the percentage of each tissue-containing field that is collagen. Tissue (red) and collagen (blue) staining areas were sampled using six micrographs for each lung as an internal control to account for variability in staining. Values for all fields were averaged to yield a single TVD per animal. TVD values per animal were then averaged to yield a group average.

Tissue for immunohistochemical analysis was de-parafinized through xylenes and a graded alcohol series. Antigen retrieval was performed by microwaving slides or three consecutive 5 min periods at 20% power in 1 mM citrate solution, pH 6.0. Slides were rehydrated in PBS, followed by blocking of endogenous peroxidases by immersion in 10% H<sub>2</sub>O<sub>2</sub> for 10 min. Slides were blocked with 5% horse serum in PBS + 4% BSA for 30 min at room temperature. Primary antibody omission and *Spp1*<sup>-/-</sup> lung tissue samples were used as negative staining controls. Tissue was stained using a goat anti-SPP1 antibody at 2  $\mu$ g/ml in 1x PBS + 4% BSA (1 h, 22°C). Detection of SPP1 was performed using a biotinylated horse-anti-goat secondary antibody at a 1:200 dilution, avidin/biotin (Vectastain Elite ABC, Vector Lab), and aminoethylcarbazole substrate. Tissue was counterstained with Mayer's hematoxylin. Lung SPP1 was quantified by color thresholding analysis using Nikon Elements analysis software.

**SPP1 Analysis in BALF and serum:** Enzyme-linked immunosorbent assay (ELISA) for SPP1 was performed using the SPP1 ELISA DuoSet (R&D Systems). The absorbance at 450 nm was read using a SpectraMax M2e plate reader (Molecular Dynamics) and standard curves and sample values were generated using SoftMax Pro software.

**qRT-PCR analysis:** Lung tissue was homogenized and total RNA isolated using TRIZOL (Sigma). Isolated RNA was treated with DnaseI and quantified by A260/A280 spectrophotometric absorbance using a BioTek Synergy 2 multimode microplate reader and Gen5 data analysis software. Two hundred nanograms of RNA was reversed transcribed using Iscript cDNA synthesis kit (BioRad) at 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 primer for either SPP1 (Mm01611440\_mH, Applied Biosystems) or 18S ribosomal RNA (RPS18) (Mm03928990\_g1, Applied Biosystems) as endogenous control. Reaction cycle is as follows: 2 min 50°C, 10 min 95°C, and then 40 cycles of 15 sec 95°C followed by 1 min 60°C. Relative levels of SPP1 transcripts was analyzed ( $\Delta\Delta C_T$ ) and expressed as log 2 fold-change.

**Lung collagen content analysis:** The right lung was excised for hydroxyproline determination via the chloramine-T spectrophotometric absorbance assay as previously described (Fattmam 2001; Woessner 1961). Tissue was placed individually into 2 ml glass ampules and dried at 110°C. After drying, 2 ml of 6N HCl was added to each ampule. Oxygen was purged from each ampule and replaced by nitrogen, vacuum-sealed and acid hydrolyzed at 110°C for 24 h. The acid was completely driven off at 110°C prior to resuspension in 2 ml of 1x PBS at 60 °C for 1 h. Rehydrated samples were centrifuged at 13,000 rpm for 10 min to remove particulate matter.

**Statistical Analysis:** All data was evaluated by one-way or two-way ANOVA as appropriate using Bonferroni's post-hoc test for multiple comparisons in each assay. Data with p values  $\leq$  0.05 were considered significant.

## Results

**SPP1 expression in response to silica treatment in male and female mice:** Increased SPP1 has been associated with pulmonary fibrosis in humans (Foster et al. 2015; Nau et al. 1997; Pardo 2005) and animal models (Berman et al. 2004; Langley et al. 2011; Ma 2012; Mangum 2004; Miyazaki et al. 1995, Oh et al. 2015; Sabo-Attwood et al. 2011; Takahashi et al. 2001). To determine whether SPP1 is also increased in silica-induced fibrosis, we exposed male and female *Spp1*<sup>+/+</sup> mice to crystalline silica and assessed SPP1 levels at 1, 3, or 14 days post-exposure. Silica increased lung SPP1 protein more in male than in female mice (Figure 1). Consistent with other reports (Ganguly et al. 2014, O'Regan 2003), SPP1 protein was detected primarily in macrophages and alveolar epithelial cells in control tissue (Figure 1A). Following silica treatment, SPP1 immunostaining was detected in the extracellular matrix, particularly in males (Figure 1C). Quantitative measurement of immunostaining of SPP1 protein (Figure 1G) and transcripts (Figure 1H) was increased more in males than females. Analysis of BALF performed post exposure indicated that silica-treated female mice had more total cells at day 3 and 14 (Figure 2A), PMN at day 3 (Figure 2C) and macrophages at day 14 (Figure 2B) than silica-treated male mice. Similar to the SPP1 immunostaining (Figure 1G) and transcripts (Figure 1H), silica-treated male mice had more BALF SPP1 protein than silica-treated female mice (Figure 2D). At day 14, serum SPP1 protein increased more in silica-treated male mice compared to silica-treated female mice (male:  $175 \pm 11$ ; female:  $149 \pm 7$  ng/ml,  $p < 0.05$ ) and silica-treated

mice more than in sex-matched saline-treated mice (male:  $116 \pm 7$ ; female:  $98 \pm 4$  ng/ml,  $p < 0.05$ ).

**BALF cells and protein recovered from *Spp1*<sup>+/+</sup> (C57BL/6J) and gene-targeted (*Spp1*<sup>-/-</sup>) mice at 14 days following silica treatment:** To further define the role of SPP1 in silica-induced lung disease, we treated *Spp1*<sup>-/-</sup> mice with silica and assessed inflammatory and fibrotic responses at day 14. BALF from silica-treated mice contained increased inflammatory cells compared to sex- and genotype-matched saline-exposed mice (Figure 3A). However, compared to silica-exposed *Spp1*<sup>+/+</sup> female mice, silica exposed *Spp1*<sup>-/-</sup> female mice had fewer total BALF cells (Figure 3A). Interestingly, the sex difference we observed previously in total cells for *Spp1*<sup>+/+</sup> mice (Brass et al. 2010) was not observed in the silica-treated *Spp1*<sup>-/-</sup> mice (Figure 3A). No differences in either macrophage or lymphocyte numbers were observed between silica-exposed male *Spp1*<sup>-/-</sup> and silica-exposed female *Spp1*<sup>-/-</sup> mice (data not shown). Also consistent with previous observations (Brass et al. 2010), silica exposure increased total BALF protein content (Figure 3B), However, among silica-exposed mice, there were no significant differences in BALF protein between sexes or genotypes.

**Lung histology, collagen, and hydroxyproline in *Spp1*<sup>+/+</sup> (C57BL/6J) and gene-targeted (*Spp1*<sup>-/-</sup>) mice 14 days following silica treatment:** In addition to an altered silica-induced BALF total cell response, male (Figure 4C) and female (Figure 4D) *Spp1*<sup>-/-</sup> mice had decreased fibrotic lesions compared to sex-matched male (Figure 4A) and female (Figure 4B) *Spp1*<sup>+/+</sup> mice after silica exposure. Similarly, lung hydroxyproline (Figure 4F) decreased in male and female *Spp1*<sup>-/-</sup> mice compared to male *Spp1*<sup>+/+</sup> mice after silica exposure. Lung hydroxyproline increased more in male *Spp1*<sup>+/+</sup> mice compared to female *Spp1*<sup>+/+</sup> mice (Figure 4F). No sex difference in lung hydroxyproline was evident between silica-treated male and female *Spp1*<sup>-/-</sup>

mice. Lung collagen was increased in silica-treated mice compared to sex- and genotype-matched saline-treated mice (Figure 4E). Female *Spp1*<sup>-/-</sup> mice had less collagen compared to male *Spp1*<sup>+/+</sup> mice after silica exposure.

**SPP1 protein expression in ovariectomized female and estrogen-treated male mice lung following silica treatment:** Based on the above observations and our previous work (Brass et al 2010), we hypothesized that the protection against silica-induced fibrosis observed in females could be due to, in part, by estrogen-mediated changes in SPP1 expression. Ovariectomized female mice had increased SPP1 immunostaining in the lung (Figure 5D and G) compared to sham female mice (Figure 5C and G), suggesting that estrogen suppresses the activation of SPP1 expression.

To confirm the role of estrogen in determining SPP1 expression and silica-induced lung fibrosis, we pre-treated male mice with estrogen for 21 days prior to silica administration. Following silica, estrogen pre-treatment resulted in increased BALF total cells, macrophages, and PMN (Figure 6A-C), decreased BALF SPP1 (Figure 6D,) and decreased lung collagen deposition (Figure 6E) and hydroxyproline (Figure 6F). These changes mirrored the increased BALF total cells (Figure 2A) and decreased BALF SPP1 (Figure 2D) and decreased collagen deposition and hydroxyproline (Fig.1-4) observed in female mice when compared to males. Together, these findings support the concept that estrogen is protective against the development of pulmonary fibrosis, perhaps through the recruitment of additional phagocytic inflammatory cells.

## Discussion

In this study, we hypothesized that sex differences between male and female mice in sensitivity to silica-induced pulmonary fibrosis are due, in part, to alterations in pro-

inflammatory and pro-fibrotic protein SPP1. Following silica exposure, SPP1 increased in lung tissue, BALF, and serum of male mice compared to female mice. Additionally, SPP1 is likely to be a pro-fibrotic mediator inasmuch as *Spp1*<sup>-/-</sup> male mice were resistant to silica-induced fibrotic lesions (Figure 4C), and hydroxyproline (Figure 4F) compared to *Spp1*<sup>+/+</sup> male mice (Figure 4A and 4F). SPP1 appears to be a sex-specific mediator as (A) the sex differences in silica-sensitivity were not evident in exposed *Spp1*<sup>-/-</sup> mice, (B) silica-induced increased SPP1 was greater in ovariectomized compared to sham female mice, and (C) silica-induced SPP1, collagen, and hydroxyproline decreased in male mice pre-treated with estrogen compared to untreated male mice. Taken together, these data suggest that estrogen-mediated repression of SPP1 expression accounts for, at least in part, the relative resistance of female mice to silica-induced fibrosis.

SPP1 contributes to human fibrotic lung disease and increases in SPP1 are associated with the pulmonary fibrosis (Foster et al. 2015; Nau et al. 1997; Pardo 2005) and the development of fibrosis in a number of animal models (Berman et al. 2004; Langley et al. 2011; Ma 2012; Mangum 2004; Miyazaki Y et al. 1995; Oh et al. 2015; Sabo-Attwood et al. 2011; Takahashi et al 2001). For example, bleomycin treatment of mice results in an increase in SPP1 mRNA and protein at 14 days post-exposure and treatment with an antibody which blocks SPP1 function partially protects against fibrosis development (Takahashi et al. 2001). Here, SPP1 protein levels are increased in lung tissue, BALF, and serum in response to silica exposure in both male and female *Spp1*<sup>+/+</sup> mice. We also observed increased SPP1 mRNA at 3 and 14 days post-exposure in silica-treated mice compared to saline-treated controls (Figure 1H). Notably, increased SPP1 mRNA has been reported in tumor necrosis factor (TNF)-induced lung fibrosis in mice (Miyazaki et al. 1995). This may be of particular importance in the pathogenesis of silica-

induced lung disease, because lung TNF is increased in silicosis patients (Vanhee et al. 1995) and silica-treated mice (Ortiz et al. 1998).

SPP1 is often described as a matricellular protein, indicating that it is localized to both the extracellular matrix and the cell cytoplasm (O'Regan 2003; Wang and Denhardt 2008). Consistent with previous reports (Takahashi et al. 2001), silica-induced SPP1 immunostaining increased in resident alveolar macrophages and alveolar epithelial cells (Figure 1C-E). These cells express both intracellular and secreted SPP1 (Ganguly et al. 2014; O'Regan 2003; Takahashi et al. 2001; Wang and Denhardt 2008). Secreted SPP1 present in lung tissue (Figure 1G) and BALF (Figure 2D) of silica-treated male mice increased more than that of silica-treated female mice. In addition, BALF SPP1 did not increase significantly in silica vs. saline female mice on days 1 and 14, but was only evident on day 3. In response to tissue injury, secreted SPP1 mediates migration, proliferation, adhesion and differentiation of fibroblasts and myofibroblasts (Lenga et al. 2008). It is also required for the proper deposition and organization of the extracellular matrix during tissue repair processes. This requirement has been noted in a variety of fibrosis models, including bleomycin-induced fibrosis, and *Spp1*<sup>-/-</sup> also have reduced collagen deposition and disorganized collagen fibrils at sites of injury (Berman et al. 2004). Consistent with these observations, lung hydroxyproline content was decreased in the *Spp1*<sup>-/-</sup> male mice compared to *Spp1*<sup>+/+</sup> male mice (Figure 4F). Recently, we reported that SPP1 promotes pneumocyte growth, and *SPP1*<sup>-/-</sup> mice have smaller, more compliant lungs with enlarged airspace (i.e., increased Lm) (Ganguly et al. 2014). Taken together, previous studies and our data suggest that lung SPP1 could contribute to fibrosis and that the sex-specific sensitivity may be due to differing levels of secreted SPP1 present in male and female lungs.

SPP1 is a known component of several fibrosis-associated pathophysiologic pathways. For example, TGFB1 essential mediator of pulmonary fibrosis and can induce SPP1 expression. Conversely, fibroblasts from *Spp1*<sup>-/-</sup> mice are insensitive to stimulation by TGFB1, indicating that SPP1 is an essential component of TGFB1 signaling in these cells (Mori et al. 2008). SPP1 contains a number of functional domains including a canonical RGD integrin-binding domain and can interact with various integrins (Sodek et al. 2000). SPP1-integrin binding induces the assembly of actin filaments in the cytoplasm, resulting in increased cell and tissue stiffness, resulting in the mechanosensitive TGFB1 activation (Henderson and Sheppard 2012). In addition, SPP1 can bind to heteromeric integrin  $\alpha v \beta 6$  (Erikson et al. 2009), the primary TGFB1-binding integrin (Munger et al. 1999; Sheppard 2015) and SPP1 may compete with TGFB1 for integrin  $\alpha v \beta 6$  binding and thus SPP1 stimulates many of the same cellular responses as TGFB1.

Our silica model has an important inflammatory component, in that female mice have increased total cells (Figure 2A on days 3 and 14), macrophage (Figure 2B on day 14), neutrophils (Figure 2C on days 3 and 14) compared to male mice following silica exposure. The role of inflammation in pulmonary fibrosis has been controversial for many years, and it still remains unclear whether anti-inflammatory therapy could be effective in treating fibrosis (Noble and Homer 2005). In this study, inflammation appears to be inversely related to the fibrotic response, suggesting that the severity of the inflammatory response may not predict the degree of fibrotic response in this model system. This observation may be influenced by the limitations of murine models. Nonetheless, animal models have been useful in uncovering specific profibrotic molecular mechanisms and pathways (Barkauskas and Noble 2014; Moore et al. 2013)). The role of macrophage and epithelial cell activation and elicited growth factors, cytokines, and other proteins, typically associated with inflammation, are still considered to be important in the

etiology of fibrosis (Wolters et al. 2014). Our current observations support the notion that the role of inflammatory cells is complex and may potentiate and resolve fibrosis.

Macrophages are thought to be the key inflammatory cell that mediates silica-induced lung injury (Leung et al. 2012). Macrophages also express high levels of SPP1 and SPP1 is thought to mediate macrophage recruitment and activation through both extracellular and intracellular pathways (Rittling 2011). Phagocytosis of particles by macrophages is the first line of defense against the inhalation of silica (or other particulates) and SPP1 can alter phagocytosis of bacteria and bacterial particles (Rittling 2011). As noted previously (Brass et al. 2010), female mice that had less fibrosis in response to silica than males also had more macrophages in their lung lavage fluid. The combination of increased numbers of macrophages and decreased SPP1 (Fig. 1 and 2) may result in greater clearance of silica particles from the lungs of female mice compared to males.

Exactly how female sex influences SPP1 expression is still unknown. One possibility is that sex-specific expression of SPP1 has a genetic basis. Several genetic variants in the human *SPP1* promoter have been identified and are functional (Giacopelli et al. 2004). One single-nucleotide polymorphism (SNP), rs28357094, is associated with decreased SPP1 expression (Giacopelli et al. 2004). Interestingly, this SNP has been associated with sex-related effects in disease severity in systemic lupus erythematosus (Kariuki et al. 2009) and juvenile dermatomyositis (Niewold et al. 2010). Although several genome-wide association studies have been conducted to identify SNPs associated with the development and progression of pulmonary fibrosis (Fingerlin et al. 2013; Mushiroda et al. 2008; Noth et al. 2013; Seibold et al. 2011), differences in SNP associations between sexes were not examined in either affected patients or controls. It is likely that the presence of a protective sex-specific SNP may have gone undetected.

Whether estrogen plays a direct or indirect role in determining sex-specific expression of SPP1 is another unanswered question. In other organ systems, *SPP1* promoter activation is estrogen-sensitive and estrogen can both stimulate (Vanacker et al. 1998; Vanacker et al. 1999) and inhibit (Arias-Loza et al. 2007; Li et al. 2000) SPP1 production depending on the cell type involved. In vascular endothelial cells estrogen can modulate *SPP1* transcription through an NFκB-mediated mechanism (Lund et al. 2009; Wang and Denhardt 2008), specifically by facilitating the formation of either transcription-activating or transcription-repressing NFκB dimers (Urtasun et al. 2012). Therefore it is possible that estrogen indirectly represses SPP1 transcription through NFκB in female mice and the lack of estrogen-mediated repression allows the excessive production of SPP1 in male mice.

In summary, we previously reported a sex-dependent response to silica-induced pulmonary fibrosis in mice (Brass et al 2010). Here we identify SPP1 as being differentially expressed in an estrogen-dependent manner and contributes, in part, to the observed sex-differences in silica-induced fibrosis between male and female mice. The relationship of sex to disease susceptibility and development is complex and is in need of further exploration. Determining how such a fundamental characteristic as sex influences disease development and outcome is essential to realize the goals of stratified medicine.

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## Figure Legends

**Figure 1.** Lung secreted phosphoprotein 1 (SPP1) expression in response to silica treatment in male and female C57BL/6J mice. Immunohistochemical staining for SPP1 in lung tissue from saline-exposed (A) male, (B) female, or silica-treated (C and E) male and (D and F) female C57BL/6J mice at 14 days post-exposure. Panels C and D are representative sections of silica-induced fibrotic lesions in male and female mice, respectively, Panels E and F are representative sections of silica-induced SPP1 expression in macrophages (arrows) and alveolar epithelial cells in male and female mice, respectively. (G) Percentage of tissue immunostaining for SPP1 protein in C57BL/6J mice at 1, 3, and 14 days post-exposure. (H) Lung SPP1 transcripts in silica-treated male and female mice in C57BL/6J mice at 1, 3, or 14 days post-exposure. Transcripts are expressed as log<sub>2</sub> fold-change compared to saline-treated, sex-matched controls and values were normalized to 18 s ribosomal protein (RPS18). SPP1 protein immunostaining increased more in male (C, E, and G) compared to female (D, F, and G) C57BL/6J mice following silica treatment. Values are means  $\pm$  SE: saline-treated groups: n = 6 mice/sex/day, silica-treated group n = 5 mice/sex/day. \* p<0.05 Silica-treated mice compared to same-sex, same-day saline-treated mice, and † p<0.05 silica-treated female mice compared to same-day silica-treated male mice determined by ANOVA with Bonferroni's correction for multiple comparisons.

**Figure 2.** Bronchoalveolar lavage fluid cells and secreted phosphoprotein 1 (SPP1) recovered from saline- or silica-treated C57BL/6J mice. (A) Total cells at day 3, and 14, (B) macrophages at day 14, and (C) neutrophils (PMN) at day 3 increased more in female mice than male mice following silica exposure. In contrast, (D) BALF SPP1 was increased more in silica exposed male mice compared to female mice at day 1, 3, and 14. Values are means  $\pm$  SE: saline-treated groups: n = 6 mice/sex/day, silica-treated groups n = 5 mice/sex/day. \* p<0.05 Silica-treated mice compared to same-sex, saline-treated mice, and † p<0.05 silica-treated female mice compared to silica-treated male mice determined by ANOVA with Bonferroni's correction for multiple comparisons.

**Figure 3.** Bronchoalveolar lavage fluid (BALF) cells and protein recovered from saline- or silica-treated *Spp1*<sup>+/+</sup> (C57BL/6J) and gene-targeted secreted phosphoprotein 1 null (*Spp1*<sup>-/-</sup>)

mice at 14 days following treatment. (A) BALF total cells decreased in silica-treated gene-targeted secreted phosphoprotein 1 null (*Spp1*<sup>-/-</sup>) female mice compared to control *Spp1*<sup>+/+</sup> (C57BL/6J) mice 14 days following silica treatment. Although increased compared to sex- and genotype-matched saline control, (B) BALF protein in silica-treated mice did not differ between sex and genotype. Values are means  $\pm$  SE: saline-treated groups: n = 6 mice/sex/genotype, silica-treated groups n = 6 mice/sex/genotype. \* p<0.05 Silica-treated mice compared to same-sex, same-genotype saline-treated mice, † p<0.05 Silica-treated female mice compared to same-genotype silica-treated male mice, and ‡ p<0.05 silica-treated *Spp1*<sup>-/-</sup> females compared to silica-treated *Spp1*<sup>+/+</sup> females determined by ANOVA with Bonferroni's correction for multiple comparisons.

**Figure 4.** Lung histology, collagen, and hydroxyproline in *Spp1*<sup>+/+</sup> (C57BL/6J) and gene-targeted secreted phosphoprotein 1 null (*Spp1*<sup>-/-</sup>) mice 14 days following silica treatment. Representative section of Masson's trichrome staining of *Spp1*<sup>+/+</sup> mouse lung from silica-exposed (A) male or (B) female mice (Bar = 100  $\mu$ m). Representative sections of Masson's trichrome staining of *Spp1*<sup>-/-</sup> mouse lung from silica-exposed (C) male or (D) female mice. Lung (E) collagen and (F) hydroxyproline, an indicator of collagen turnover/deposition, in silica-treated mice at 14 days post-exposure. In silica-exposed male *Spp1*<sup>+/+</sup> mice, (A) histological fibrotic lesions and (F) hydroxyproline were greater than in silica-exposed (B and F) *Spp1*<sup>+/+</sup> female mice. Fibrotic lesions and hydroxyproline were also less in (C and F) and *Spp1*<sup>-/-</sup> male or in (D and F) *Spp1*<sup>-/-</sup> female mice compared to male in (A and F) *Spp1*<sup>+/+</sup> mice. Values are means  $\pm$  SE: saline-treated groups: n = 6 mice/sex/genotype, silica-treated groups n = 6 mice/sex/genotype. \* p<0.05 Silica-treated mice compared to same-gender, same-genotype saline-treated mice, and † p<0.05 Silica-treated mice compared to silica-treated *Spp1*<sup>+/+</sup> males determined by ANOVA with Bonferroni's correction for multiple comparisons

**Figure 5.** Secreted phosphoprotein 1 (SPP1) protein expression in sham and ovariectomized (OVX) female C57BL/6 mice following 14 days silica treatment. Representative lung immunostaining SPP1 in saline-exposed (A) sham or (B) OVX female mice or silica-treated (C) sham or (D) OVX female mice. Representative primary antibody exclusion controls in (E) sham or (F) OVX female mice following 14 day silica treatment. (G) Percentage of tissue SPP1 immunostaining. Following silica treatment, histological fibrotic lesions and SPP1

immunostaining increased in OVX mice compared to sham female mice. Saline-treated groups: n = 6 mice/sex/day, silica-treated groups n = 6 mice/sex/day. \* p<0.05 Silica-treated mice compared to group-matched saline-treated mice, and † p<0.05 vs. Silica-treated OVX mice compared to silica-treated sham mice determined by ANOVA with Bonferroni's correction for multiple comparisons.

**Figure 6.** Bronchoalveolar lavage fluid (BALF) cells and secreted phosphoprotein 1 (SPP1) in vehicle-treated male and estrogen-treated (E<sub>2</sub>) male C57BL/6J mice at 14 days post-exposure silica treatment. Silica-induced BALF (A) total cells, (B) macrophages, (C) neutrophils (PMN), increased and (D) SPP1 protein decreased with estrogen treatment. Lung (E) collagen, and (F) hydroxyproline were decreased by estrogen treatment. Values are means ± SE: saline-treated groups: n = 6 mice/sex/day, silica-treated groups n = 5 mice/sex/day. \* p<0.05 vs. same-gender vehicle (olive oil) control, † p<0.05 saline silica-treated males determined by ANOVA followed by Bonferroni's correction for multiple comparisons.

Figure 1

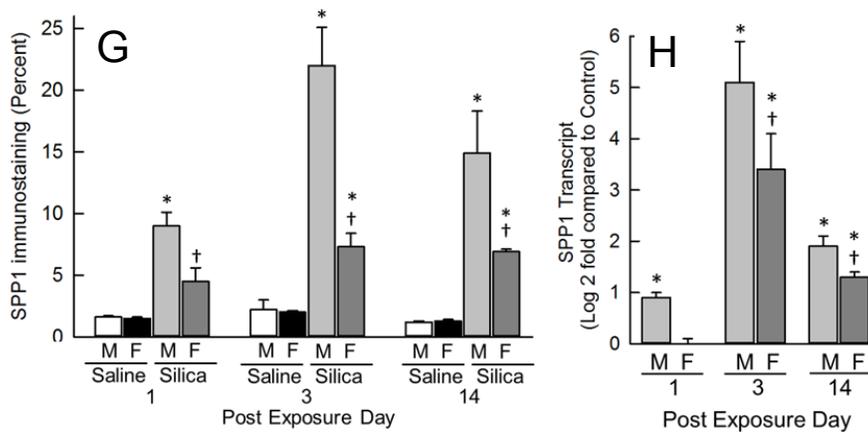
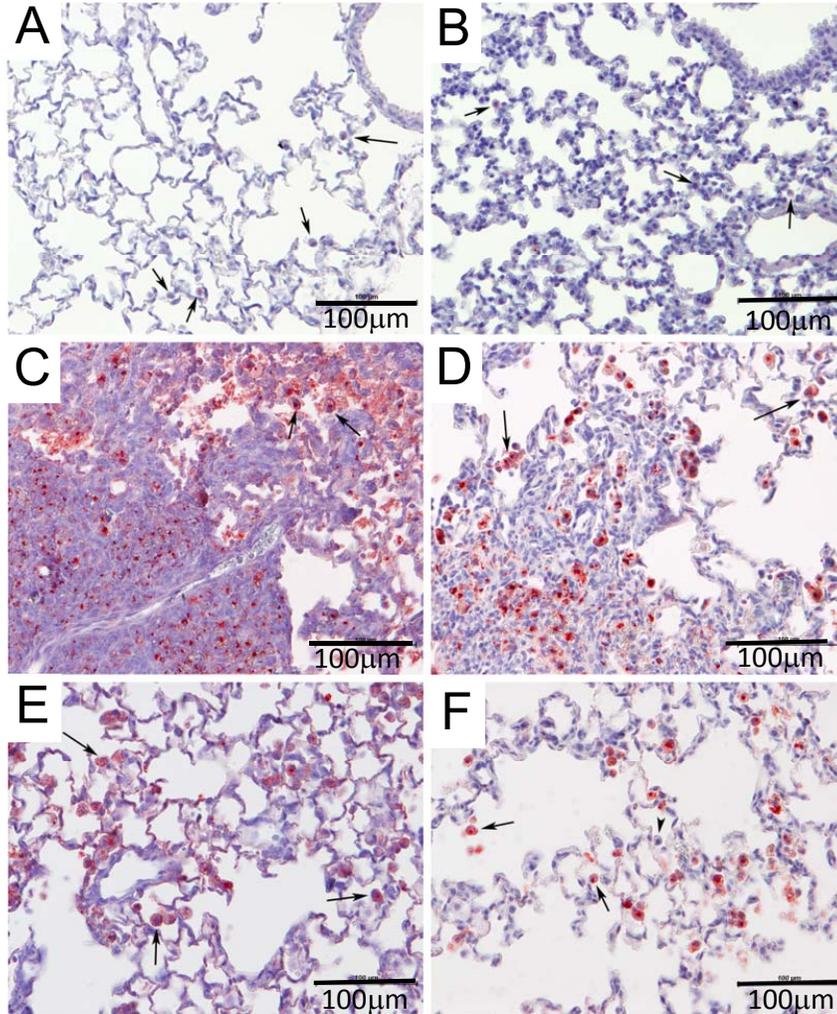


Figure 2

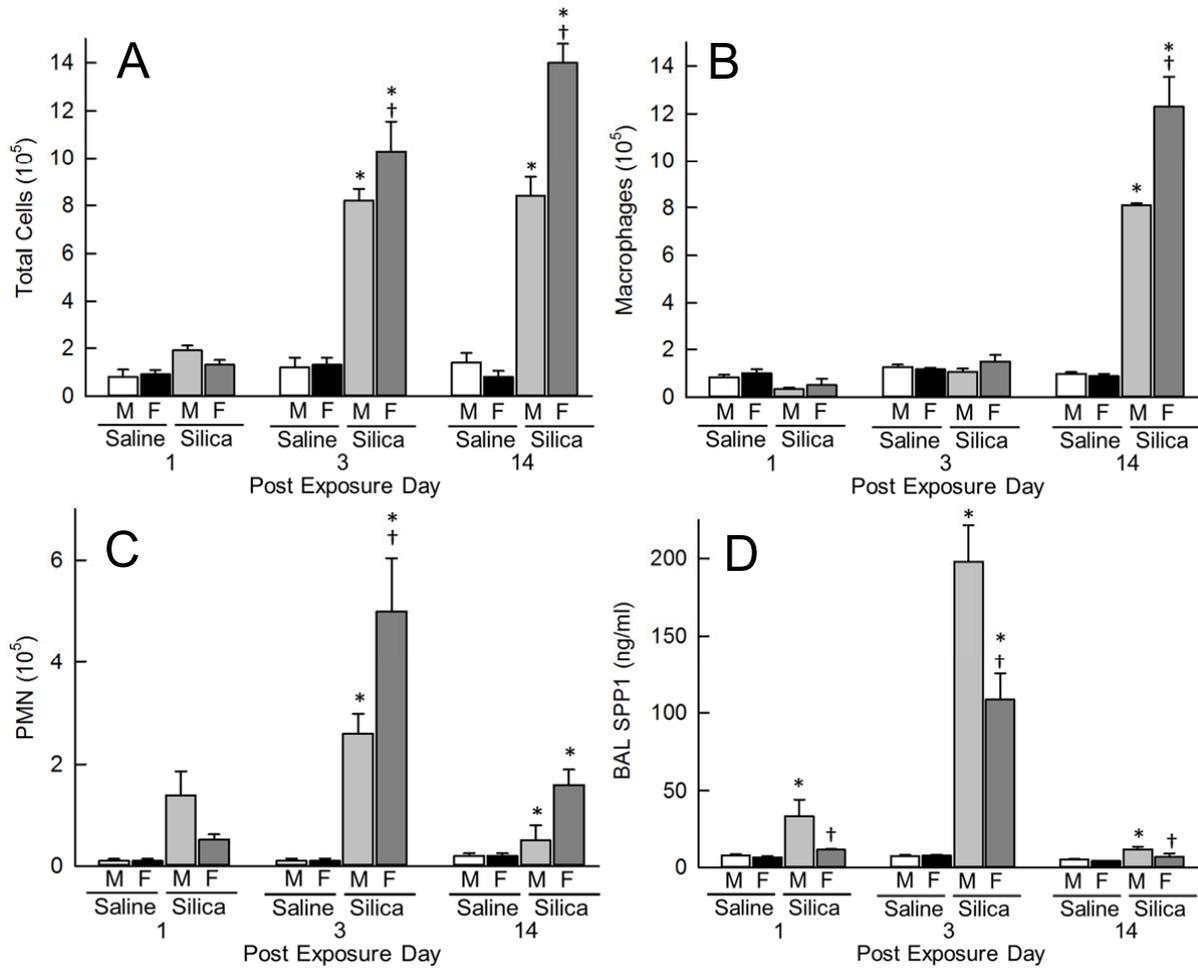


Figure 3

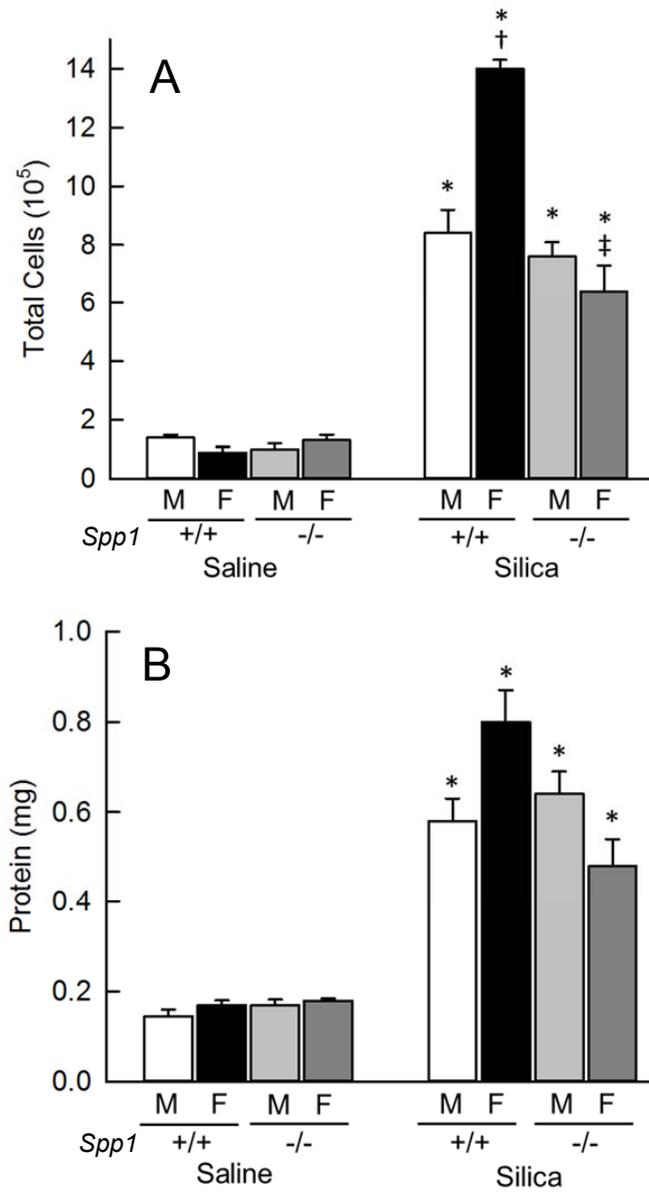


Figure 4

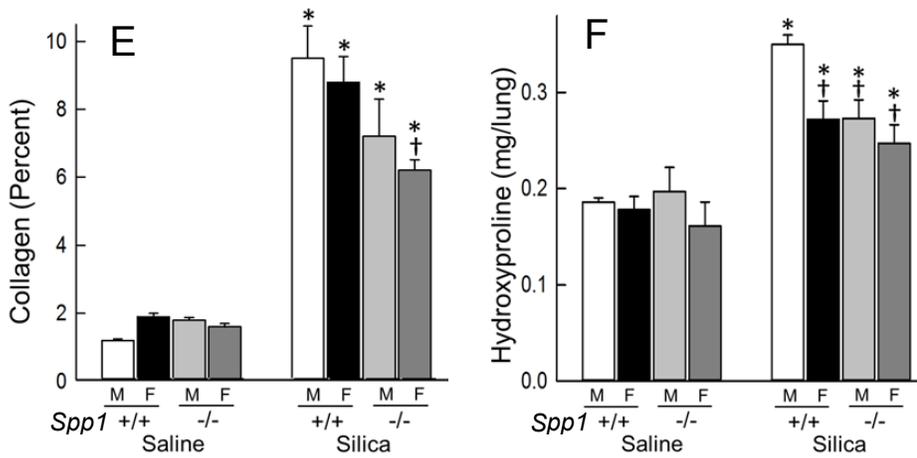
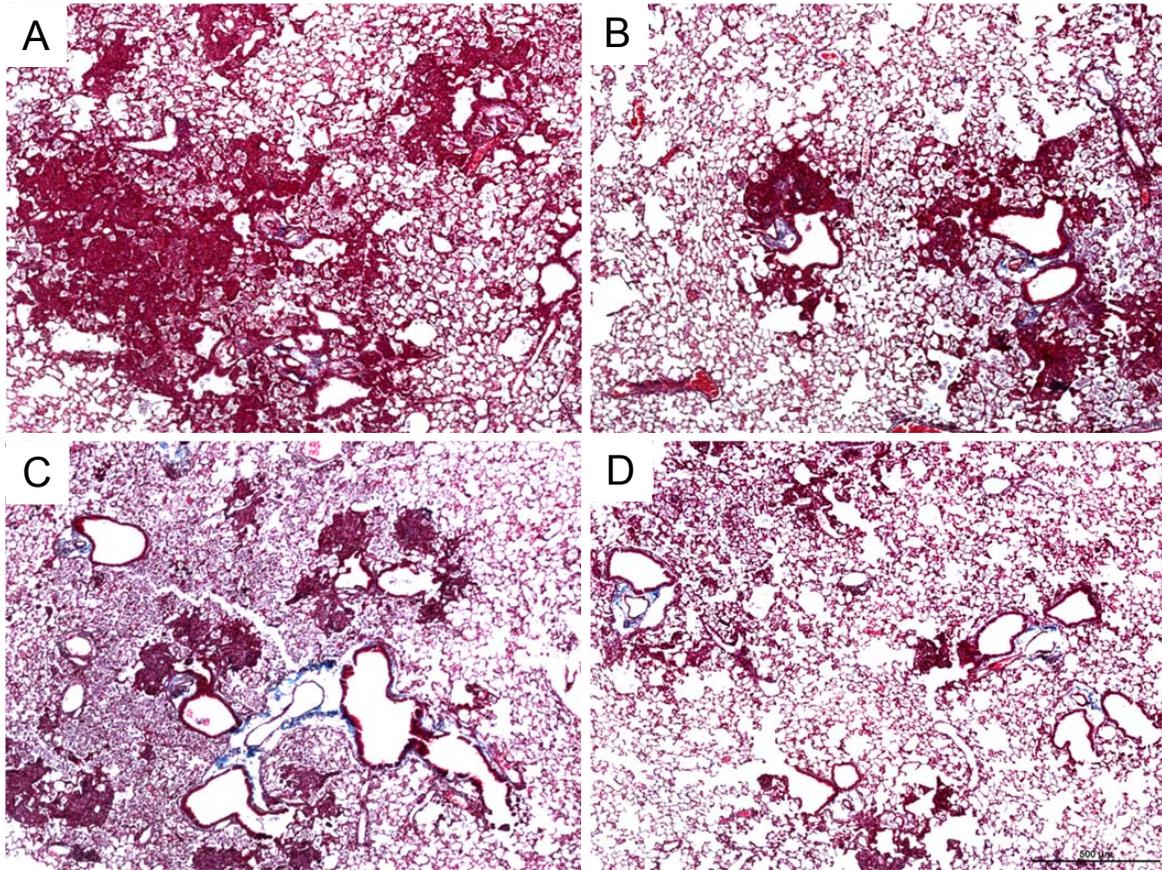


Figure 5

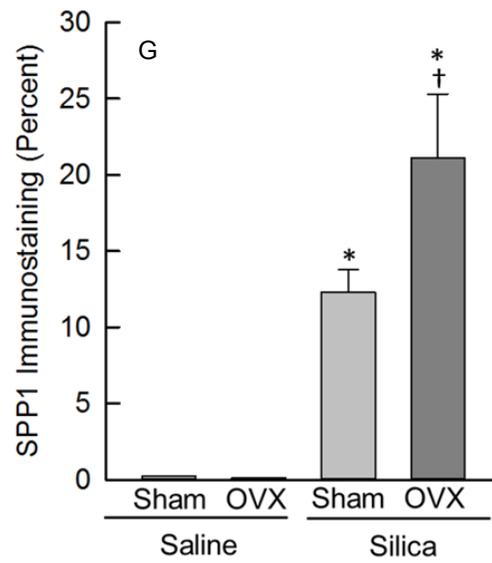
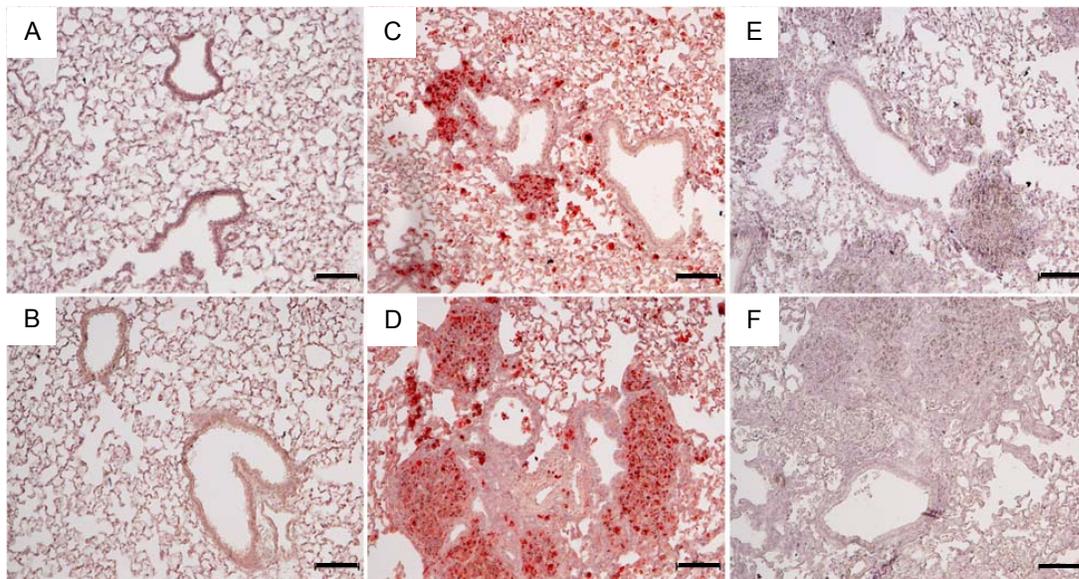


Figure 6

