Silica-Directed Mast Cell Activation Is Enhanced by Scavenger Receptors

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Inhalation of crystalline silica results in pulmonary fibrosis and silicosis. It has been suggested that mast cells play a role in these conditions. How mast cells would influence pathology is unknown. We thus explored mast cell interactions with silica *in vitro* and in B6.Cg-Kit^{W-sh} mast cell-deficient mice. B6.Cg-Kit^{W-sh} mice did not develop inflammation or significant collagen deposition after instillation of silica, while C57Bl/6 wild-type mice did have these findings. Given this supporting evidence of a role for mast cells in the development of silicosis, we examined the ability of silica to activate mouse bone marrow-derived mast cells (BMMC), including degranulation (β -hexosaminidase release); production of reactive oxygen species (ROS) and inflammatory mediators; and the effects of silica on Fc ϵ RI-dependent activation. Silica did not induce mast cell degranulation. However, TNF- α , IL-13, monocyte chemoattractant protein-1, protease activity, and production of ROS were dose-dependently increased after silica exposure, and production was enhanced after Fc ϵ RI stimulation. This mast cell activation was inhibited by anti-inflammatory compounds. As silica mediates some effects in macrophages through scavenger receptors (SRs), we first determined that mast cells express scavenger receptors; then explored the involvement of SR-A and macrophage receptor with collagenous structure (MARCO). Silica-induced ROS formation, apoptosis, and TNF- α production were reduced in BMMC obtained from SR-A, MARCO, and SR-A/MARCO knockout mice. These findings demonstrate that silica directs mast cell production of inflammatory mediators, in part through SRs, providing insight into critical events in the pathogenesis and potential therapeutic targets in silicosis.

Keywords: B6.Cg-Kit^{W-sh} sash mouse; CD204; macrophage receptor with collagenous structure; mast cell; silicosis; SR-A

Among environmental exposures that lead to pathologic changes in tissues is crystalline silica, which can result in occupational silicosis from inhalation of silica dusts in manufacturing, construction, farming, and mining operations. Silicosis, for which there is no effective treatment, leads to decreased pulmonary function and increased susceptibility to diseases of the respiratory tract (1, 2).

Involvement of mast cells in silica-induced pulmonary inflammation has been suggested by two clinical observations. First, the number of mast cells within the lungs of individuals exposed to silica dust is increased (3). Second, an increase in mast cells staining for basic fibroblast growth factor located within silicotic nodules has been reported in lung sections obtained from patients with silicosis (4). Despite the suggestive

CLINICAL RELEVANCE

This study demonstrates a role for mast cells in silicosis and is the first study to examine direct effects of silica on mast cell biology. It provides evidence that treatment of silicosis should explore mast cells as a potential therapeutic target.

evidence for a role of mast cells in the development of silicosis, there are no systematic studies of the effects of silica on mast cells.

Before entering into experiments testing the hypothesis that silica can direct mast cell activation, a critical role for mast cells in promoting silicosis was first verified in mast cell-deficient sash mice (B6.Cg-Kit^{W-sh}) after exposure to silica. Because the mast cell-deficient mice did not develop silicosis, while mice with normal mast cells did develop pathology, we entered into experiments to determine the ability of silica to directly activate mast cells. Cultured bone marrow-derived murine mast cells (BMMC) were exposed to silica, followed by analysis of cell viability, for effects on degranulation, and for the production of reactive oxygen species (ROS), cytokines, and proteolytic activity. The possible role of scavenger receptor class A (SR-A) and macrophage receptor with collagenous structure (MARCO) was explored in the genesis of positive responses to silica exposure. As will be shown, mast cells exposed to silica respond primarily by production of cytokines, the synthesis of which can be inhibited with anti-inflammatory agents and is enhanced by scavenger receptors.

MATERIALS AND METHODS

Cell Culture and Silica Treatment

Mouse BMMC were cultured from femoral marrow cells of C57Bl/6 wild-type, SR-A KO, MARCO KO, and SR-A/MARCO double knockout (KO) mice. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 25 mM HEPES, 1.0 mM sodium pyruvate, nonessential amino acids (BioSource International, Camarillo, CA), 0.0035% 2-ME, and 300 ng/ml recombinant mouse IL-3 (PeproTech, Rocky Hill, NJ). BMMC were used after 4–6 wk of culture. Silica (Min-U-Sil-5, with an average particle size of 1.5–2 μ m) was obtained from Pennsylvania Glass Sand Corporation (Pittsburgh, PA) and was acid washed, dried, and determined to be free of endotoxin. Cells were treated with silica in 96-well plates at 0, 6.25, 12.5, 25, or 50 μ g/cm². Water-soluble dexamethasone (Sigma-Aldrich, St. Louis, MO) was used at 10⁻⁶ M and was added to BMMC cultures 1 h before the addition of silica.

Mice

Mast cell-deficient sash mice (B6.Cg-Kit^{W-sh}) and wild-type (C57Bl/6) mice were obtained from Jackson Laboratories at 4–6 wk of age (Bar Harbor, ME). Mice were anesthetized with tribromoethanol (0.1 mg/kg) before intranasal instillation of either 30 μ l sterile saline or a 30 μ l sterile saline suspension of 1 mg crystalline silica. All mice received two instillations 2 wk apart. Six mice were instilled per group, and the

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four groups consisted of C57Bl/6 wild-type mice or B6.Cg-*kit*^{W-sh} mast cell-deficient mice instilled with either saline or silica. Mice were killed 3 mo after the first instillation of silica.

SR-AI/II KO mice and MARCO KO mice were generously provided by Dr. L. Kobzick of the Harvard School of Public Health (Boston, MA) (5, 6). Both knockouts were backcrossed for at least eight generations to the C57Bl/6 background. SR-A I/II/MARCO double KO mice were generated in the laboratory of Dr. L. Kobzick by intercross of the single SR-A I/II and MARCO knockouts. Founding C57Bl/6 wild-type mice were purchased from Jackson Laboratories. Mice used in these studies were propagated by homozygous mating colonies and maintained in microisolator units within the University of Montana specific pathogen-free animal facility. Cages, bedding, and food were sterilized by autoclaving, and mice were handled with aseptic forceps or aseptic gloves. Mice were allowed food and water *ad libitum* and were used experimentally between 6 and 8 wk of age. All animal use procedures were in accordance with National Institutes of Health and approved by the University of Montana institutional animal care and use committee.

Identification of SR-A I/II and MARCO KO Mice

DNA samples were extracted from mouse tail and amplified using forward (5'-CAAGTGATACATCTCAAGGTC-3'), reverse (5'-CTG TAGATTCACGGACTCTG-3'), and Neo insert (5'-GAGGAGTA GAAGGTGGCGCGAA-3') primers (Operon Technologies, Alameda, CA) encompassing the site of the SR-A I/II and Neo insert (7). For MARCO identification, DNA samples were amplified using the following primers: MARCO WT, forward (5'-CAGCTGGGTCCATACCAGC-3') and reverse (5'-CTGGAGAGCCTCGTTGACC-3'); and MARCO KO, forward (5'-CCACGCTCATCGATAATTTCAC-3') and reverse (5'-CCTGCAGTGGCCGTCGTTTAA-3'). Amplification was performed in a PTC-200 Gradient Cycler (MJ Research, Las Vegas, NV) with the following parameters: 1 min 94°C, 1 min 60°C, 1 min 72°C and subjected to 35 cycles of PCR. Genotyping was determined by separation of the amplified PCR products by 1.5% agarose gel electrophoresis at 75 V for 2.5 h. C57Bl/6 WT mice show a 440-bp band, whereas SR-A I/II KO mice show a 325-bp band. MARCO WT primers produce a 500-bp band and MARCO KO primers produce an 850-bp band.

Lung Histology

Three months after either saline or silica instillation, C57Bl/6 wild-type and B6.Cg-*kit*^{W-sh} mast cell-deficient mice were given a lethal dose of sodium pentobarbital. Lungs were then perfused with 1–2 ml Histochoice fixative (Amresco, Solon, OH), embedded in paraffin blocks and sectioned at 5 μ m, and mounted on glass slides (American Histolabs, Inc, Gaithersburg, MD) (8). Sections were stained with Gomori's trichrome, hematoxylin and eosin (H&E), and toluidine blue (American Histolabs).

Cell Viability and Apoptosis

Cell viability was determined by trypan blue staining and lactate dehydrogenase (LDH) release (9). The percentage of LDH release was measured in supernatants of BMMC exposed to silica for 24 h (Bio-Vision, Mountain View, CA). Untreated cells were used as a negative control and 1%-triton X-100 (Sigma-Aldrich)-treated BMMC were used as a positive control, representing 100% LDH release. Apoptosis was determined by DNA fragmentation using a TiterTacs TUNEL Assay (R&D Systems, Minneapolis, MN) (10). Equal numbers of cells (1×10^5 /well) were plated in a 96-well plate, and silica was added for 24 h at 6.25, 12.5, 25, or 50 μ g/cm². Nontreated cells were used as a negative control and a DNA nuclease-generated positive control was used. Experiments were repeated three times. The reported values are mean optical density (OD) values from each treatment.

Degranulation and Cytokine Release

BMMC were seeded at 5×10^4 cells/well in 96-well flat-bottom plates and sensitized with 100 ng/ml mouse IgE anti-DNP (Sigma-Aldrich) for 24 h for degranulation experiments (11). For treated samples, silica was added at 0, 6.25, 12.5, 25, or 50 μ g/cm² for 24 h before addition of increasing concentrations of DNP-HSA (0–1000 ng/ml) (Sigma-Aldrich). After 30 min incubation of antigen at 37°C, *p*-nitrophenyl-*N*-acetyl- β -D-glucopyranoside was added to cell supernatants and lysates

for 90 min as a chromogenic substrate for *N*-acetyl- β -D-hexosaminidase (Sigma-Aldrich) (11). The reaction was stopped with 0.2 M glycine. Optical density was measured at 405 nm using a GENios ELISA plate reader (ReTirSoft, Inc., Toronto, ON, Canada). β -Hexosaminidase release was expressed as the percentage of total cell content after subtracting background release from unstimulated cells. Cytokines were measured in cell culture supernatant of BMMC seeded at 2×10^5 cells/well for 24 h after addition of 6.25, 12.5, 25, or 50 μ g/cm² silica. For experiments with silica exposure and Fc ϵ RI stimulation, BMMC were exposed to silica for a total of 24 h at 0–50 μ g/cm² and sensitized overnight with 100 ng/ml IgE anti-DNP (Sigma-Aldrich). DNP-HSA (Sigma-Aldrich) was added at 100 ng/ml for a total of 8 h before supernatant collection for cytokine analysis. Mouse TNF- α , IL-13, and monocyte chemoattractant protein (MCP)-1 (CCL2) were measured using Quantikine ELISA kits (R&D Systems, Minneapolis, MN).

Protease Assay

Silica-exposed BMMC were examined for protease activity using a Quantileave Protease Assay Kit (Pierce, Rockford, IL). Briefly, supernatants from BMMC exposed to 0, 6.25, 12.5, 25, or 50 μ g/cm² silica for 24 h were collected and analyzed for the ability to cleave succinylated casein. A control blank without casein from each sample was run in parallel and subtracted as background. Experiments were repeated three times. The reported values are mean OD values measured at 450 nm.

Reactive Oxygen Species Detection

ROS were measured by a 96-well plate assay employing the fluorescent probe dichlorofluorescein (DCF) (12). BMMC (1×10^6 /ml) were incubated with DCF diacetate (20 μ M) in cell culture medium for 15 min at 4°C with rotation. Cells were then washed in HEPES buffer (10 ml) and seeded at 200,000 per well in a black opaque 96-well microplate. Zileuton (Cayman Chemical, Ann Arbor, MI) and Trolox (EMD Biosciences, San Diego, CA) (10 μ l) were added and DCF fluorescence monitored for 5 min before the addition of silica. After addition of silica, DCF fluorescence was then monitored for a further 10 min using a GENios fluorescent plate reader (ReTirSoft Inc.) set at an excitation wavelength of 492 nm and emission wavelength of 535 nm. Fluorescence was expressed as relative fluorescent units (RFU). The kinetic data was collected using an XFlour4 macro within Microsoft Excel.

RT-PCR

BMMC (1×10^6 cells/condition) from C57Bl/6 mice were exposed to silica for 30 min, 1 h, or 2 h at 0, 6.25, 12.5, 25, or 50 μ g/cm². Cells were collected and total mRNA was isolated using QIAshredders and RNeasy Mini Kits (Qiagen, Valencia, CA). RNA was transcribed to cDNA using a Reaction Ready First Strand cDNA synthesis kit (Superarray, Frederick, MD). Gene-specific primers for SR-A I (MSR1), SR-A II (MSR2), MARCO, and glyceraldehyde phosphate dehydrogenase (GAPDH) were purchased from Superarray and transcripts were amplified using a Reaction Ready Hotstart "Sweet" PCR kit according to manufacturer's instructions (Superarray).

Statistics

Statistical analysis employed the software package PRISM, version 4 (GraphPad, San Diego, CA). Differences between untreated and silica-treated samples were assessed using one-way ANOVA with Bonferroni post-test. Area under the curve (AUC) was calculated for ROS measurements using PRISM. All values are reported as means $\pm$ SEM.

RESULTS

Mast Cell-Deficient Mice Fail to Develop Inflammation and Extensive Collagen Deposition after Silica Exposure

To assess the role of mast cells in the development of silicosis, mast cell-deficient mice (B6.Cg-*Kit*^{W-sh}) were administered two 1-mg instillations of silica, with effects on lung histology examined 3 mo after the first instillation of silica. As determined by Gomori's trichrome staining, silica-exposed wild-type mice demonstrated increased collagen deposition within the lung as compared with saline-instilled wild-type mice (compare Figure 1A

Figure 1. Silica induces collagen deposition in C57Bl/6 wild-type, but not B6.Cg-*Kit*^{W-sh} mast cell-deficient mouse lungs 3 mo after two intranasal instillations of 1 mg silica. C57Bl/6 mice were instilled with either saline (A) or silica (C). B6.Cg-*Kit*^{W-sh} received instillations of either saline (B) or silica (D). Lungs were stained with Gomori's trichrome stain to examine collagen deposition. Images were obtained at $\times 5$ magnification and are representative of six mice per group.

with 1C). By H&E staining, wild-type mice were found to have developed granulomatous lesions typical of silicotic lesions, alveolar thickening, and pronounced inflammatory infiltrates within the interstitial and alveolar spaces (compare Figure 2A with 2B). In contrast, mast cell-deficient mice exposed to silica did not

develop granulomatous lesions, alveolar thickening, or any significant inflammation or collagen deposition (compare Figures 1D and 2D with 1C and 2C). There were no differences in the number of lung mast cells in silica-exposed wild-type mice as compared with saline controls (data not shown). As expected,

Figure 2. Silica induces inflammation in C57Bl/6 wild-type, but not B6.Cg-*Kit*^{W-sh} mast cell-deficient mouse lungs 3 mo after two intranasal instillations of 1 mg silica. C57Bl/6 mice were instilled with either saline (A) or silica (C). B6.Cg-*Kit*^{W-sh} received instillations of either saline (B) or silica (D). Lungs were stained with H&E to examine inflammation. Images were obtained at $\times 5$ magnification and are representative of six mice per group.

no mast cells were detectable in B6.Cg-*Kit^{W-sh}* mast cell-deficient mouse lungs, and no mast cells were observed after silica exposure (data not shown). These observations confirm a role for mast cells in silicosis.

Silica Induces Apoptosis in BMMC but Has Minimal Effect on Degranulation

A series of experiments was then initiated to determine the effects of silica on cultured mast cells. At the start of these experiments, BMMC were visually examined after exposure to silica for 24 h. An apparent internalization of silica particles was observed (see Figures 3A and 3B). This was not unexpected, as mast cells are known to be phagocytic and macrophages similarly internalize silica (13, 14).

Silica exposure has been reported to induce cytotoxicity (apoptosis and necrosis) in alveolar macrophages and thus contribute to tissue inflammation (15–18). As shown in Figure 4A, silica did induce between 5 and 15% LDH release from BMMC exposed to 6.25 $\mu\text{g}/\text{cm}^2$ silica, and at 50 $\mu\text{g}/\text{cm}^2$ silica, between 20 and 40% LDH release was observed. To further examine the ability of silica to affect viability of BMMC, the TUNEL assay was also employed. Figure 4B shows a dose-dependent increase in cells staining positive for DNA fragmentation as determined by an

in vitro TUNEL assay. Significant differences between untreated BMMC and 12.5, 25, and 50 $\mu\text{g}/\text{cm}^2$ silica were seen 24 h after exposure (Figure 4B, $P \leq 0.05$). Consistent with LDH release (Figure 4A), silica induces apoptosis in BMMC at higher doses. However, by either assay, a majority of the cells remained viable 24 h after silica exposure.

Silica exposure was next examined for the ability to alter Fc ϵ RI-mediated degranulation of BMMC as one mechanism by which silica might enhance inflammation mediated by mast cells. Pretreatment of BMMC (sensitized with mouse IgE anti-DNP) with silica at doses of 6.25, 25, and 50 $\mu\text{g}/\text{cm}^2$ minimally decreased β -hexosaminidase release after addition of increasing amounts of DNP-HSA compared with BMMC that were not exposed to silica (Figure 4C). Silica exposure by itself similarly did not induce β -hexosaminidase release from mouse BMMC (data not shown). These results reveal that silica has little indirect effect on Fc ϵ RI-mediated mast cell degranulation, nor does silica directly induce degranulation.

Silica Induces Production of Reactive Oxygen Species in BMMC

Silica exposure induces the production of ROS in macrophages and has been implicated in mediating biological effects such as fibrosis after silica exposure (19). Therefore, the ability of mast cells to produce ROS after silica exposure was assessed. Figure 4D (*insert*) shows that induction of ROS occurs rapidly and is sustained after addition of silica. Total ROS production measured as area under the curve in silica-exposed BMMC was significantly increased after exposure to 12.5, 25, and 50 $\mu\text{g}/\text{cm}^2$ silica compared with untreated BMMC (Figure 4D).

Silica Induces Cytokine and Chemokine Production and Enhances IgE-Mediated Cytokine and Chemokine Production in BMMC

Silicosis has been associated with increased production of profibrotic cytokines and chemokines in bronchoalveolar lavage fluid, including TNF- α , IL-13, and MCP-1 (CCL2) (20–22). Therefore, the ability of silica to directly induce production of such mediators from BMMC, as well as the ability of silica to enhance Fc ϵ RI-mediated cytokine and chemokine production, was explored. After 24 h of silica exposure, the production of TNF- α , IL-13, and MCP-1 by BMMC are dose-dependently increased (Figures 5A–5C, respectively). Silica-exposed BMMC that were sensitized with IgE anti-DNP and challenged with DNP-HSA for 8 h showed enhanced production of TNF- α , IL-13, and MCP-1 (Figures 5D–5F).

In addition to cytokine production, protease activity was measured in the supernatant of silica-exposed BMMC. The overall protease activity within the supernatant from BMMC exposed to silica at doses of 12.5, 25, and 50 $\mu\text{g}/\text{cm}^2$ for 24 h was significantly increased as compared with untreated BMMC (Figure 6).

Mast Cells Express Scavenger Receptors: Apoptosis and ROS Production Is Reduced in SR-A $^{-}$, MARCO $^{-}$, and SR-A/MARCO-Deficient BMMC

Silica has been reported in part to mediate effects through SR-A on macrophages (15, 16). To determine if mast cells also express scavenger receptors, we examined these cells for the presence of mRNA for these receptors. Figure 7 demonstrates mRNA expression for two closely related scavenger receptors, SR-A I/II and MARCO, both before and after silica exposure in BMMC. While mRNA for SR-A II (MSR2) was increased after silica exposure over a time course, all scavenger receptors appeared to increase by 2 h after silica exposure ranging from 6.25–50 $\mu\text{g}/\text{cm}^2$ (Figures 7A

Figure 3. Silica interacts with and is internalized by BMMC. Images of (A) untreated or (B) BMMC exposed to 25 $\mu\text{g}/\text{cm}^2$ silica for 24 h and stained with Wright-Giemsa (magnification: $\times 100$). Arrows indicate silica particles.

Figure 4. Silica induces LDH release, apoptosis, and ROS production, but has little effect on β -hexosaminidase release in BMMC. (A) LDH release was measured in supernatant of silica-exposed BMMC. (B) Apoptosis was determined by DNA fragmentation in BMMC (1×10^5 /well) exposed to 0, 6.25, 12.5, 25, or 50 $\mu\text{g}/\text{cm}^2$ silica for 24 h. (C) Fc ϵ RI-mediated β -hexosaminidase release, as a measure of degranulation, was determined 24 h after exposure of BMMC (50,000/well) to 0, 6.25, 12.5, 25, or 50 $\mu\text{g}/\text{cm}^2$ silica. (D) ROS production in silica-exposed BMMC where silica was added to BMMC 5 min after addition of the fluorescent probe DCF, at doses of 0, 6.25, 12.5, 25, or 50 $\mu\text{g}/\text{cm}^2$. Intracellular ROS production was measured up to 30 min (insert in D). Area under the curve of silica-induced ROS production with ionomycin as a positive control (D). Data are means $\pm$ SEM for three experiments. * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$ as compared with untreated BMMC.

and 7B). Because of this evidence that mast cells express scavenger receptors, experiments were entered into examining the effects of silica on mast cells cultured from scavenger receptor KO mice.

As shown earlier (Figure 4B), silica exposure at 50 $\mu\text{g}/\text{cm}^2$ induces DNA fragmentation as compared with untreated cells. Therefore, the induction of apoptosis by silica in BMMC derived from SR-A I/II KO, MARCO KO, and SR-A/MARCO double KO mice was examined. As shown in Figure 8A, there was a significant decrease in the level of DNA fragmentation in silica-exposed BMMC from MARCO KO and SR-A/MARCO double KO mice when compared with silica-exposed BMMC from C57Bl/6 mice. ROS production in BMMC from SR-A KO, MARCO KO, and SR-A/MARCO double KO mice was similarly less compared with ROS production by BMMC from C57Bl/6 controls after exposure to 50 $\mu\text{g}/\text{cm}^2$ silica (Figures 8B and 8C). These results indicate that scavenger receptors are able to mediate in part the apoptotic state of BMMC, as well as ROS production, in response to silica.

TNF- α Production Is Altered in Silica-Exposed BMMC from SR-A- and MARCO-Deficient Mice

Due to the changes in apoptosis and redox states observed in SR-A- and MARCO-deficient BMMC after silica exposure, the role of these scavenger receptors in mediating TNF- α production after silica exposure was examined. Figure 8D shows a role for SR-A in enhancing cytokine production after silica exposure. TNF- α is significantly decreased after 50 $\mu\text{g}/\text{cm}^2$ silica exposure in the SR-A KO and SR-A/MARCO double KO BMMC as compared with WT BMMC exposed to the same dose of silica (Figure 8D, $P \leq 0.01$).

Treatment of BMMC with Zileuton, Trolox, and Dexamethasone Reduced Silica-Directed Mast Cell Activation

There is currently no effective treatment for silicosis; however, steroids and anti-inflammatory agents have been used in an attempt to control symptoms and the progression of disease. Using anti-inflammatory and antioxidant compounds, the ability

Figure 5. Silica directs TNF- α , IL-13, and MCP-1 production and enhances Fc ϵ RI-mediated production of these mediators in BMMC. BMMC (2×10^5 /well) were exposed to 0, 6.25, 12.5, 25, or 50 $\mu\text{g}/\text{cm}^2$ silica for 24 h before the measurement of TNF- α (A), IL-13 (B), and MCP-1 (C). In addition, BMMC sensitized with IgE anti-DNP and exposed overnight to 0, 6.25, 12.5, 25, or 50 $\mu\text{g}/\text{cm}^2$ silica before challenge with DNP-HSA for 8 h were measured for production of TNF- α (D), IL-13 (E), and MCP-1 (F). Data are means $\pm$ SEM for three independent experiments. * $P \leq 0.05$ and *** $P \leq 0.001$ as compared with untreated BMMC.

of these agents to inhibit silica-directed mast cell activation *in vitro* was examined. Using the 5-lipoxygenase inhibitor Zileuton (Figure 9A), or the vitamin E derivative Trolox (Figure 9B), we were able to significantly reduce ROS levels in BMMC after silica exposure. Figure 9C shows total area under the curve for the blockade of ROS using both Zileuton and Trolox. Measured as AUC, Zileuton added at 20 μM to BMMC immediately before the addition of silica significantly inhibited ROS formation as compared with BMMC treated with 50 $\mu\text{g}/\text{cm}^2$ silica (Figure 9C).

Figure 6. Silica induces release of proteases from BMMC. Protease activity was measured in supernatants of BMMC exposed to 0, 6.25, 12.5, 25, or 50 $\mu\text{g}/\text{cm}^2$ silica for 24 h. Data are means $\pm$ SEM for three independent experiments. * $P \leq 0.05$ and *** $P \leq 0.001$ as compared with untreated BMMC.

Also, the addition of 300 μM Trolox to BMMC before the addition of silica significantly reduced ROS levels as compared with BMMC treated with 50 $\mu\text{g}/\text{cm}^2$ silica (Figure 9C).

BMMC that were treated with dexamethasone for 1 h before the addition of silica significantly inhibited TNF- α production (Figure 9D). As previously shown, silica dose-dependently increased TNF- α production from BMMC 24 h after exposure; however, using dexamethasone, silica-induced TNF- α production at all doses of silica was abrogated (Figure 9D). As a control, dexamethasone was also used to inhibit Fc ϵ RI-mediated TNF- α production from BMMC (Figure 9D). Although similar treatments have been attempted in patients with silicosis, these data suggest that silica-directed mast cell activation could be inhibited using a combination of clinically relevant anti-inflammatory compounds.

DISCUSSION

A role for mast cells in silica-induced pulmonary inflammation has been suggested; however, possible mechanisms in this process have not been explored (3, 4, 23). In this article we demonstrate that B6.Cg-*kit*^{W-sh} mast cell-deficient mice did not develop silicosis, including significant collagen deposition, 3 mo after silica exposure. Further *in vitro* experiments on the effects of silica on mast cells suggest that one reason may be that silica has the ability to directly activate mast cells, a process in part dependent on scavenger receptors, here shown for the first time to be expressed on mast cells. Thus, silica-exposed mast cells produced cytokines, generated ROS, and released proteolytic activity.

Figure 7. Demonstration that BMMC express mRNA for SR-A (MSR1 and MSR2) and MARCO. (A) BMMC (1×10^6 cells/condition) were examined for mRNA expression of SR-A and MARCO in untreated or BMMC exposed to 50 µg/cm² silica for 30 min, 1 h, or 2 h. (B) In addition, mRNA for SR-A and MARCO were examined in BMMC at 2 h after addition of 0, 6.25, 12.5, 25, or 50 µg/cm² silica. GAPDH was used as a control for constitutive gene expression. Results are representative examples of three separate experiments.

Mast cells have been implicated in the development of pulmonary inflammation 2 wk after exposure to silica in that WBB6F₁-W/W^v mast cell-deficient mice were relatively protected from acute exposure to silica compared with wild-type mice (23). Because responses were examined 14 d after silica exposure, inflammation, but not the development of fibrosis, could be determined (23). Thus wild-type mice exhibited lung infiltrates, an increase in lung mast cells, and neutrophilia in the bronchoalveolar lavage fluid (23). Lung lesions and neutrophilia were less pronounced in W/W^v mice (23). A problem in concluding mast cells were important to the evolution of inflammation after silica exposure in W/W^v mice is that mast cell deficiency in W/W^v mice is accompanied by some immune deficits and by thrombocytopenia (24).

These latter concerns have led us to use a second and more recent mast cell-deficient mouse model, employing sash mice (B6.Cg-*kit*^{W-sh}) to examine effects of silica exposure on pulmonary tissues. We also chose to examine the chronic rather than acute response to silica so we could also determine chronic effects of the inflammatory response, including the development of fibrosis. We found that in B6.Cg-*kit*^{W-sh} mast cell-deficient mice, both inflammation and collagen deposition were minimal 3 mo after silica instillation (Figures 1 and 2). However, wild-type mice developed pathology which resembled that seen in patients with silicosis, including formation of silicotic lesions and collagen deposition. Future experiments, as possible given the duration of this protocol, will attempt to examine the ability to restore the silicosis response in mast cell-deficient mice through reconstitution of lung mast cells. For now, we focused our attention to how silica could directly alter mast cell biology.

Silica has been reported to induce apoptosis of alveolar macrophages and a dysregulation of apoptosis due to silica exposure has been implicated in disease processes including inflammation, fibrosis and autoimmunity (8, 25–30). For example, *in vivo* treatment of silica-exposed mice with caspase inhibitors has been reported to significantly inhibit neutrophil accumulation and alleviate pulmonary inflammation (27). While internalization of silica in BMMC was observed, the majority of BMMC did remain viable 24 h after silica exposure as measured by LDH release and DNA fragmentation (Figures 4A and 4B). Although these data do suggest that some silica effects on mast cells and inflammation may occur via apoptosis, since the majority of BMMC remain viable, silica-exposed BMMC could well contribute to

inflammation by additional mechanisms requiring responsive cells.

Other environmental contaminants, such as certain heavy metals, have been reported to enhance FcεRI-mediated mast cell degranulation, a mast cell response associated with allergic disease (31). Therefore, the ability of silica to alter IgE-dependent BMMC degranulation was assessed. As Figure 4C demonstrates, silica had little effect on FcεRI-mediated degranulation. Silica by itself did not induce BMMC degranulation.

Silica has been reported to induce ROS in alveolar macrophages, and this in turn has been reported to enhance inflammation (19, 21, 32, 33). In this study, silica exposure dose-dependently induced ROS production in BMMC. This induction was rapid and sustained (Figure 4D). However, in this study ROS production was largely intracellular. Thus, it seems plausible that silica-induced ROS by mast cells is not contributing significantly to tissue damage.

One probable mechanism for mast cell involvement in silicosis is through alteration of cytokine or chemokine levels within the lung. Therefore, the ability of silica to induce TNF-α, IL-13, and MCP-1, all reported to be major contributors to the development of pulmonary inflammation and fibrosis, was examined (21, 22, 26, 34–38). Figure 5 demonstrates that silica exposure dose-dependently increased TNF-α, IL-13, and MCP-1. These mediators contribute to inflammation through the recruitment of additional inflammatory cells such as neutrophils or lymphocytes (39). In addition, TNF receptor KO mice have been reported to have impaired responses to silica exposure, including development of silicosis which has been attributed to alterations in chemokine levels, including MCP-1 (32, 38, 40). IL-13 has been studied extensively as a major contributor to the development of pulmonary fibrosis in several different models (35, 37, 41–43). Mouse BMMC produced significant quantities of IL-13 after silica exposure and may represent a major source of IL-13 in silica-induced pulmonary inflammation.

The ability of silica to alter FcεRI-mediated cytokine production was also assessed (Figure 5). BMMC exposed to silica for 24 h before stimulation via FcεRI aggregation-enhanced production of TNF-α, IL-13, and MCP-1 as compared with BMMC stimulated via FcεRI but not exposed to silica. However, these effects appear to be no more than additive and therefore silica exposure does not appear to alter FcεRI-mediated signaling events, consistent with results examining degranulation.

Figure 8. Apoptosis, ROS, and TNF- α production are reduced in silica exposed BMMC cultured from SR-A/II KO, MARCO KO, or SR-A/MARCO double KO mice. (A) DNA fragmentation was measured in BMMC obtained from scavenger receptor KO mice and exposed to 50 $\mu\text{g}/\text{cm}^2$ silica for 24 h. Data are presented as the percentage decrease in OD values as compared with C57Bl/6 WT BMMC exposed to 50 $\mu\text{g}/\text{cm}^2$ silica. (B) ROS production using the fluorescent probe DCF, following exposure to 50 $\mu\text{g}/\text{cm}^2$ silica in SR-A KO, MARCO KO and SR-A/MARCO double KO BMMC is shown over 25 min. (C) ROS production was also analyzed as AUC in BMMC from scavenger receptor KO and WT mice exposed to 50 $\mu\text{g}/\text{cm}^2$ silica. (D) TNF- α production measured by ELISA is shown 24 h after addition of 50 $\mu\text{g}/\text{cm}^2$ silica in SR-A KO, MARCO KO, and SR-A/MARCO double KO BMMC (silica doses presented in a linear form for representation only). Data are presented as means $\pm$ SEM ($n = 3$). * $P \leq 0.05$ and ** $P \leq 0.01$ as compared with wild-type BMMC exposed to the same dose of silica.

In addition to cytokine production, an increase in the protease activity was measured within the supernatant of silica-exposed BMMC (Figure 6). The production of mast cell proteases may be another important mechanism in the development of silicosis since mast cell chymase has been reported to be involved in a bleomycin-induced model of pulmonary fibrosis by mediating the cleavage of TGF- β 1 to its active form (44). In addition to chymase, mast cell tryptase has been reported to be involved in activation of fibroblasts and stimulation of collagen production (45–47).

The recognition of silica by macrophages and their subsequent activation has been reported to occur via scavenger receptors (15, 16, 48, 49). The expression of, and a functional role for, these scavenger receptors has not been reported when considering mast cells. Therefore, mRNA expression of SR-A on BMMC, as well as a closely related receptor, MARCO, was first examined. Figure 6 shows mRNA for scavenger receptors in BMMC and that silica exposure increased mRNA levels for

SR-A II (MSR2) within 2 h. Thus BMMC had mRNA for both SR-A I (MSR1) and MARCO; however, only SR-A II message appeared to increase after silica exposure.

We were thus able to use BMMC from mice deficient in SR-A I/II, MARCO, or both, to demonstrate that these receptors modulate some of the effects of silica on mast cells (Figure 7). Apoptosis, ROS, and TNF- α production were decreased in MARCO KO and SR-A/MARCO double KO BMMC after silica exposure. In contrast to other reports, it appears that MARCO is playing a more prominent role in enhancing cellular responses to silica in mast cells compared with SR-A I/II (15, 16, 48). This is consistent with reports that MARCO is more important in binding of TiO₂ and unopsonized bacteria in alveolar macrophages than is SR-A I/II (6, 50). These data demonstrate that scavenger receptors enhance the response to silica in BMMC. Whether additional scavenger receptors not yet identified are also involved remains a possibility.

Figure 9. Treatment of silica exposed BMMC with Zileuton and Trolox reduces ROS formation and treatment with dexamethasone inhibits silica-directed TNF- α production. (A) Zileuton, a 5-lipoxygenase inhibitor, was added to BMMC at concentrations of 2, 10, and 20 μM 5 min before addition of 50 $\mu\text{g}/\text{cm}^2$ silica to inhibit ROS formation. (B) In addition, the vitamin E derivative Trolox was added at concentrations of 30, 100, and 300 μM 5 min before addition of 50 $\mu\text{g}/\text{cm}^2$ silica to inhibit intracellular ROS formation. (C) Area under the curve was calculated for the reduction of silica-induced ROS formation using Zileuton and Trolox. (D) TNF- α was measured in BMMC ($2 \times 10^5/\text{well}$) that were treated with dexamethasone (10^{-6} M) 1 h before the addition of 50 $\mu\text{g}/\text{cm}^2$ silica for 24 h. BMMC that were sensitized overnight with IgE anti-DNP and challenged for 8 h with 100 ng/ml DNP-HSA were used as a control for the inhibition of TNF- α production by dexamethasone. Data are means $\pm$ SEM for three independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$ as compared with silica-exposed BMMC not treated with either Zileuton, Trolox, or dexamethasone. ## $P \leq 0.01$ and ### $P \leq 0.001$, silica-exposed BMMC treated with dexamethasone as compared with silica-exposed BMMC.

Finally, the ability to inhibit silica-directed mast cell activation was examined using several anti-inflammatory and antioxidant compounds including Zileuton (5-lipoxygenase inhibitor), Trolox (vitamin E derivative), and dexamethasone. Both Zileuton and Trolox were able to reduce ROS formation in BMMC after silica exposure, and dexamethasone was able to significantly inhibit silica-directed TNF- α production from BMMC (Figure 9). Although similar treatments have been explored for silicosis, they have largely not been effective because the disease is not detected until late stages. However, these data suggest that several clinically relevant compounds that in part target mast cells may be beneficial in the treatment of silicosis if the disease was detected early.

In total, the data presented demonstrate that silica can direct mast cell activation leading to production of inflammatory mediators, and that these responses are enhanced by mast cell scavenger receptors. Through this process, mast cells appear to have a significant role in the recruitment of inflammatory cells leading

to the development of silicosis. Taken together, these data provide evidence that treatment of silicosis and possibly other particle-induced inflammatory conditions should explore the mast cell as an important therapeutic target.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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