Airway Delivery of Silica Increases Susceptibility to Mycobacterial Infection in Mice: Potential Role of Repopulating Macrophages[1]

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Silica exposure results in an increased lifelong risk of developing mycobacterial pulmonary infections. To date, there are no animal models that replicate this finding to permit assessment of the mechanisms underlying susceptibility to mycobacterial infection. To test the hypothesis that prior silica exposure increases risk of mycobacterial infection, we intratracheally (I.T.) administered silica, a control dust (Al_2O_3) or saline into mechanically ventilated C57BL/6 mice. Later, the mice received *Mycobacterium avium* or *Mycobacterium tuberculosis* I.T. Mice were sacrificed at defined time points and mycobacteria in lung homogenates were quantified. *M. avium* or *M. tuberculosis* infection was markedly increased in silica-exposed mice compared with mice exposed to either Al_2O_3 or saline beginning 3 wk after silica exposure. Similarly, lung sections from silica-exposed mice had many more acid fast bacilli$^+$ (AFB$^+$) organisms than from control mice. Alveolar macrophages (AMs) from bronchoalveolar lavage of silica-exposed mice also revealed a higher number of mycobacteria compared with mice treated with Al_2O_3 or saline. In addition, passive transfer of AMs from silica-exposed mice to control mice increased *M. tuberculosis* susceptibility. These results indicate that silica exposure converts mycobacteria-resistant mice into mycobacteria-susceptible mice via a process that likely involves a new population of AMs that are more susceptible to mycobacterial infection. ***The Journal of Immunology,*** **2009, 182: 7102–7109.**

Silicosis is a fibrotic lung disease caused by inhalation of crystalline silica particles (SiO_2) (1) that continues to be a major public health problem worldwide. Exposure to silica can result in acute silicosis, accelerated silicosis, or chronic silicosis (2, 3). Silica exposure is also well recognized as a major risk factor for the development of mycobacterial infections including tuberculosis (4–6). About one-half of the mycobacterial infections diagnosed in silica-exposed individuals are caused by *Mycobacterium tuberculosis* with the remainder caused by *Mycobacterium kansasii* and *Mycobacterium avium* intracellulare (7–9). There is some evidence that the magnitude of silica exposure impacts susceptibility to mycobacterial infection with the incidence of tuberculosis developing in subjects with advanced silicosis being higher than those with minimal exposure to silica (4). However, even individuals with minimal silica exposure have an increased risk of developing tuberculosis (10). Although the epidemiologic link between silica exposure and mycobacterial infection is well established, the mechanisms by which silica predisposes individuals to an increased susceptibility to mycobacterial infection remain unknown. Advances in this understanding have been limited by the lack of an animal model by which to study this interrelationship.

The interaction of mycobacteria and alveolar macrophages (AMs)[3] in the lung is a critical step in the control of the mycobacterial infections. AMs use lysozymal enzymes, reactive oxygen intermediates, and reactive nitrogen intermediates to rid the lung of mycobacterial infection (11–13). However, in situations where this initial response fails, viable bacilli can survive and replicate, leading to progressive pulmonary infection (14). A number of factors that decrease macrophage functions have also been identified to greatly increase the risk of developing tuberculosis and other mycobacterial infections. These include HIV infection, malnutrition, age, and alcohol (15–18).

Silica particles have direct effects on AMs (19) and, depending on the concentration, alter key metabolic functions and/or decrease antibacterial defenses (20), stimulate release of various inflammatory mediators (20, 21), and alter AM phagocytosis (22). Silica has also been shown to disrupt calcium homeostasis (23), increase oxygen radical production, and induce DNA damage in AMs (24, 25). At high concentrations in vitro, silica is directly toxic to AMs resulting in cell injury or death (21, 26). Previous evidence suggests that in addition to a membranolytic effect, silica may also induce apoptosis in AMs (20). Because silica administration impairs or kills macrophages (22), it has been suggested that silica damage to AMs results in impaired host defense against mycobacteria infection and accounts for the increased risk for mycobacterial disease in individuals exposed to silica (4–6, 27–30).

Animal models are an important tool for investigating pathogenesis of infection. However, there are currently no in vivo models of silica exposure that demonstrate increased risk of mycobacterial infections. The objective of the current study was to determine whether silica exposure leads to increased susceptibility to mycobacterial infection. We now provide evidence that mycobacterial infection is markedly increased in silica-exposed mice compared to control mice. In addition, the passive transfer of AMs

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[3] Abbreviations used in this paper: AM, alveolar macrophage; BAL, bronchoalveolar lavage; I.T., intratracheal(ly); EGFP, enhanced GFP; AFB, acid fast bacilli.

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harvested from silica-exposed mice after 4 wk to control mice increases *M. tuberculosis* susceptibility. These results demonstrate for the first time that silica exposure increases susceptibility to mycobacterial infection that is likely mediated by a new population of macrophages appearing weeks after the initial silica exposure.

Materials and Methods

Animals

C57BL/6 mice, 6–8 wk old, were purchased from The Jackson Laboratory and housed under pathogen-free conditions at the animal facility of the University of Cincinnati, College of Medicine. All animal procedures were approved by the University of Cincinnati Institutional Animal Care and Use Committee. The mice were housed in a barrier facility, maintained in sterile conditions in microisolator cages, and supplied with sterilized food and water. C57BL/6 mice were chosen for this study, because they are considered to be relatively resistant to infection with *M. avium* and *M. tuberculosis* (31, 32).

All experimental animals were maintained in microisolator cages until use. Animals were infected under BSL2 conditions with *M. avium*. Alternatively, they were transferred to the BSL3 animal facility at the University of Cincinnati and infected with *M. tuberculosis* in a class III biohazard safety cabinet.

Culture and isolation of mycobacteria

M. avium (strain 25291), obtained from American Type Culture Collection, was grown at 37°C in Middlebrook 7H9 broth (Difco) supplemented with 10% ADC enrichment (5% BSA fraction V, 2% dextrose, and 0.003% catalase) and 0.05% Tween 80 or on agar Middlebrook 7H11 medium (Difco) supplemented with OADC (0.05% oleic acid, 5% BSA fraction V, 2% dextrose, 0.004% beef catalase, and 0.85% $NaCl_2$). On a regular basis, mycobacterial purity was verified by: 1) Kinyoun acid fast smears, 2) culture of organisms in 7H11 Middlebrook selective medium for 6–8 wk and examination for the appearance of characteristic colonies, and 3) Gram stain and subculturing on blood agar to exclude presence of contaminating fungi or other bacteria.

In addition to *M. avium*, we studied the virulent strain of *M. tuberculosis* (Erdman, ATCC 35801). GFP-expressing H37Rv was a gift from Dr. V. Deretic (University of New Mexico, Albuquerque, NM). *M. tuberculosis* organisms were cultivated on 7H11 agar plates and harvested in RPMI 1640 containing 10 mM HEPES to form predominantly single-cell suspensions. To minimize problems with clumping, suspensions were gently sonicated in a GenProbe bath sonicator. The accuracy of counts was confirmed with a serial dilution and determination of CFUs.

Isolation of murine AMs

The AMs were isolated as previously described by our laboratory (33). Briefly, mice were sacrificed and lavaged using HBSS without Ca^{2+} or Mg^{2+} plus 0.6 mM EDTA. Approximately 10 ml of bronchoalveolar lavage (BAL) fluid was obtained from each mouse. The BAL fluid was centrifuged (1500 × *g*, 5 min), the cells were resuspended in lysing solution containing 0.01 M $KHCO_3$ with 0.15 M NH_4Cl, centrifuged (1500 × *g*, 5 min) to remove RBC, and resuspended in 0.9% NaCl. Cell counts were determined using a hemocytometer. Cell differential was determined by examination of cytopreparation smears (Cytospin II; Shandon Southern Instruments) after staining with the Hema 3 staining system (Fisher Scientific). Viability of the AMs was >98% as determined by trypan blue exclusion.

Passive transfer of AMs into recipient mice

Mice were anesthetized with isoflurane, after which the trachea was exposed using blunt dissection. A small incision was made in between the tracheal rings and a 20-gauge angiocath was inserted into the trachea. The AMs isolated from saline- or silica-administered mice (5×10^5) in 50 μl of HBSS were administered into the lungs via the tracheal catheter. The AM suspension was followed by 200 μl of air to clear the angiocath and upper respiratory tract of the mice. After 10 min, the mice were administered *M. tuberculosis* intratracheally (I.T.). Mice were sacrificed after 30 days and *M. tuberculosis* in the lung homogenates was assessed by determination of CFU.

Silica exposure

Crystalline silica was obtained in the form of alpha quartz (MinUSil 5; US Silica). Previous and recent studies from our laboratory have shown that silica crystals are directly cytotoxic to rat AMs, and the degree of cytotoxicity is dependent on the size of the particles (0.5–5 μm), their concentration as well as the duration of exposure (34–36). This preparation was used for all in vivo experiments. Before use, the silica was heated to 250°C for 2 h to destroy endotoxin (35, 36). For in vivo exposure, 6- to 8-wk-old pathogen-free mice (20–25 g) from The Jackson Laboratory were anesthetized with isoflurane and I.T. inoculated with 0.05 ml of sterile saline or 0.05 ml of saline containing 5 mg of silica/mouse (250 mg/kg of the body weight) or 5 mg of Al_2O_3/mouse (Sigma-Aldrich) via a catheter (37, 38). Al_2O_3 (mean crystal size of 2.4 μm) was used as a nontoxic inorganic crystal control (negative control) (39).

Infection of mice with M. avium *and* M. tuberculosis

Mice were infected I.T. with *M. avium* (5×10^5–5×10^8) suspended in 50 μl of HBSS via the tracheal catheter. The mice were ventilated for 10 min and then allowed to recover. Thirty days later, the mice were sacrificed and *M. avium* were recovered from lung homogenates.

Similarly *M. tuberculosis* (2.5×10^1–1×10^6) suspended in 50 μl of HBSS was administered I.T. into the lungs of mice in the BSL3 facility of the University of Cincinnati. Thirty days after *M. tuberculosis* administration, mice were sacrificed and the burden of *M. tuberculosis* was determined from lung homogenates.

Determination of mycobacterial CFUs in the lung

To assess mycobacterial growth, the lungs were removed aseptically at specified time points. The lungs were cut into small pieces and homogenized. Viable mycobacteria in the lung homogenates were then assessed as CFUs by performing serial dilutions from the lung homogenate and plating onto 7H11 agar in 6-well plates in duplicates. The plates were placed in CO_2-permeable sealed Ziploc bags (Minigrip lab guard; Seguin) and incubated at 37°C with 5% CO_2 for 2–3 wk and colonies were counted. The plates were again incubated for an additional 2 wk to decrease the possibility of failure to detect slower growing *M. tuberculosis* strains. No difference in the number of colonies was observed in reincubated plates.

Histologic examination of mouse lungs

Mice were sacrificed and the lungs were surgically removed, fixed with 10% neutral-buffered formalin, and paraffin embedded. Lung sections (5 μm) were stained using either H&E or Ziehl-Neelsen staining. Histopathologic changes were examined using light microscopy.

Viability of AMs

The viability of AMs was determined by a viability/cytotoxic kit obtained from Molecular Probes (catalog no. L3224). This is a two-color fluorescence-based color assay, which simultaneously identifies live vs dead cells on the basis of membrane integrity. A volume of 50 μl of live/dead assay reagent was added to AMs (5×10^4) obtained from mice exposed to silica, Al_2O_3, or saline and incubated at 37°C for 30 min in the dark. Following incubation, the AMs were transferred onto a slide and viewed under fluorescent microscopy. The images were taken randomly from the slide. The percentage of live cells was calculated as the number of live cells divided by the number of total cells at each time point. The number of live cells over total number of cells gives the live:dead ratio.

Analysis of silica-induced apoptosis in vivo

Morphologic analysis of apoptotic AMs was performed using an annexin V-binding assay. The annexin V assay is based on translocation of phosphatidylserine from the inner (cytoplasmic) leaflet of the plasma membrane to the outer (cell surface) leaflet soon after the induction of apoptosis, and that the annexin V protein has a strong, specific affinity for phosphatidylserine. AMs were isolated from the BAL on days 3, 7, 14, 21, and 30 days after silica exposure. BAL cells (5×10^6) were stained with annexin V using an Annexin-V-FLUOS staining kit (Roche Diagnostics) according to the manufacturer's instructions. The samples were divided into two. Apoptotic morphology was determined using a Spot cam fluorescence microscope (Zeiss) and photographed using a digital camera. The AMs from five to seven microscopic fields were counted per area. Approximately 200–300 cells per group were assessed in randomly selected fields to avoid experimental bias. The percentage of apoptotic cells was calculated by determining the number of cells with the stain in the total number of examined cells. The cells were also analyzed (10,000 cells/sample) on a FACScan (BD Biosciences) by using CellQuest flow cytometric analysis software.

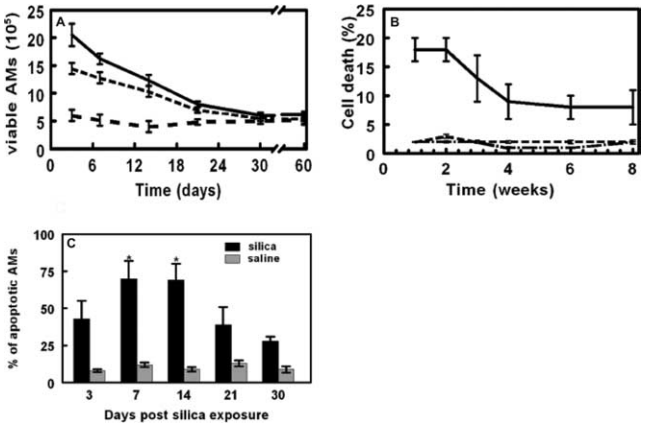

FIGURE 1. Effect of silica on AM numbers. Silica or saline were instilled I.T. into recipient C57BL/6 mice. *A*, The AM number retrieved by BAL was demonstrated after 1, 2, 3, 4, and 8 wk by cell differentials and was normalized by live/dead staining. Data are expressed as viable AMs. These data indicate that silica increases in AM number for ~3 wk as indicated by the dashed line (- - -). Total BAL cells were also increased following silica administration as indicated by the solid line (—); non-AM cells were largely neutrophils that returned to normal levels by 3 wk (data not shown). *B*, Silica (solid line), Al_2O_3 (dashed line), and saline (dotted line) were instilled I.T. into recipient C57BL/6 mice and the viability of AMs was assessed at 1, 2, 3, 4, and 8 wk by live/dead staining. Data are expressed as dead cells of the percentage of total cells. These data indicate that silica results in immediate injury to the AMs but is significantly diminished by 4 wk ($p < 0.001$). *C*, AMs were isolated by BAL on 3, 7, 14, 21, and 30 days after silica exposure. AMs were incubated with annexin V and assessed morphologically by fluorescence microscopy. The percentage of apoptotic cells was calculated by determining the number of cells with annexin V relative to the total number of cells. The results are expressed as the mean of three experiments performed in triplicate from four mice at each time point. ∗, Significantly different from saline control at $p < 0.05$.

Statistical analysis

All experiments were conducted in triplicate with a minimum of three experiments. The results are expressed as means ± SEM. The statistical difference between the control and experimental data was determined by Student's *t* test or ANOVA with paired comparisons. A $p < 0.05$ was considered to be statistically significant (40).

Results

Effects of silica on AMs

To examine the effect of silica exposure on the airway cells, mice were exposed to silica, Al_2O_3, or saline and the cells were retrieved by BAL at different time points (3 days and 1, 2, 3, 4, and 8 wk). At 3 days after silica exposure, a marked increase in airway AM was observed that returned to baseline by ~3 wk (Fig. 1*A*). Cell viability of AMs was assessed by live/dead staining and also indicated an increase in nonviable cells in the airway at 3 days after silica exposure which decreased, but remained elevated for at least 8 wk (Fig. 1*B*). We also assessed to what extent silica induced apoptosis in the AM population. Fig. 1*C* shows the percentage of total BAL AMs that were apoptotic in mice exposed to silica for 3, 7, 14, 21, and 30 days of exposure. Relative to control mice, beginning at the earliest time point examined (3 days), silica exposure markedly increased the percentage of apoptotic AM, peaking at days 7–14 and then falling back toward the control levels over the subsequent weeks. This was further confirmed by FACS analysis (data not shown). These data indicate that silica administration results in a rapid increase in BAL cells, including AM. The number of AMs returns to control levels by 4 wk, but elevated levels of nonviable and apoptotic cells persisted beyond that time.

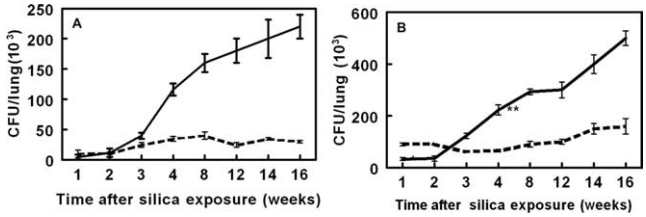

FIGURE 2. Susceptibility to mycobacterial infection as a function of time after silica exposure. C57BL/6 mice were administered silica or saline I.T. and after 1, 2, 3, 4, 6, 8, 10, 12, 14, or 16 wk, groups of mice received *M. avium* (10^6) or *M. tuberculosis* (2.5×10^1). Mice were sacrificed 30 days after infection. The extent of *M. avium* infection (*A*) or *M. tuberculosis* (*B*) infection was determined by lung CFUs. In silica-treated mice, CFUs for *M. tuberculosis* were initially lower than control levels as indicated by the solid line (—), a time of maximal injury to AMs (Fig. 1*A*). However, after 3–4 wk, *M. tuberculosis* CFUs revealed a 10-fold increase in silica-treated mice above control levels as indicated by dashed line (- - -). The *y*-axis in each panel is the number (CFUs) $\times 10^3$ of bacilli (*M. avium* and *M. tuberculosis*), one single time point (30 days) postinfection for each group. The *x*-axis in each panel is the time after silica exposure in which infection with the mycobacteria took place. This is not a growth curve of the bacteria. The results are expressed as the mean ± SEM of three experiments performed in triplicate from four mice at each time point and are representative of two independent experiments. (∗, $p < 0.05$ saline vs silica (1–2 wk)).The CFU data indicate that 4 wk after silica exposure is the critical time point for increased susceptibility to mycobacterial infection.

Silica exposure increases murine susceptibility to mycobacterial infection

We next investigated the kinetics of silica-induced susceptibility to mycobacterial infection in vivo by varying the time course of silica exposure and determining susceptibility to *M. avium* and *M. tuberculosis* infection. Silica or saline was administered I.T. to C57BL/6 mice. At 1, 2, 3, 4, 6, 8, 10, 12, 14, or 16 wk after silica exposure, subgroups of mice were infected I.T. with *M. avium* (10^6). Mice were sacrificed 30 days following mycobacterial administration, lungs were homogenized, and the number of *M. avium* was determined and expressed as CFUs. For each time point tested, silica-exposed/*M. avium*-infected mice were compared with mice receiving saline/*M. avium*. When infection with *M. avium* occurred within 1–2 wk after silica exposure, there was no significant difference in *M. avium* growth within the lungs relative to mice that had received saline instead of silica. However, beginning 3 wk after silica exposure, mice exposed to silica showed a significant increase in *M. avium* infection compared with the group that had received saline ($p < 0.05$ comparison of silica with saline exposure; Fig. 2*A*). Furthermore, the capacity of prior silica exposure to increase the *M. avium* CFUs within the lung remained evident for up to 16 wk after silica exposure, when the experiment was terminated. At all time points tested, *M. avium* growth remained stable in C57BL/6 mice administered with saline. These CFU data indicate that 4 wk following silica exposure is the critical time point for increased susceptibility of mice to *M. avium* infection. Consequently, this 4-wk time point was used for the subsequent *M. avium* experiments.

To determine the effect of silica exposure on susceptibility to *M. tuberculosis* infection, mice received *M. tuberculosis* (Erdman, 2.5×10^1) 1, 2, 3, 4, 6, 8, 10, or 12 wk after silica exposure. Thirty days later, CFUs were determined. Interestingly, the levels of *M. tuberculosis* at 30 days were lower in the mice infected at weeks 1 and 2 after silica exposure compared with control mice ($p < 0.05$, both comparisons; Fig. 2*B*). This decreased susceptibility to

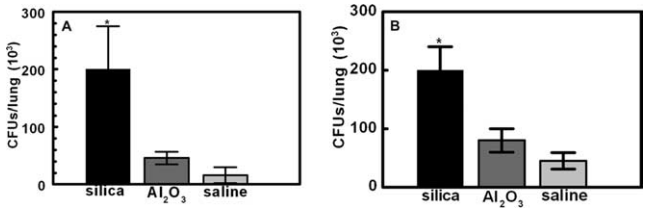

FIGURE 3. Susceptibility to mycobacteria infection is specific to silica exposure. C57BL/6 mice were I.T. administered saline, Al_2O_3, or silica and, after 30 days, all mice received *M. avium* (10^6) or *M. tuberculosis* (2.5×10^1). After 30 days (60 days from silica exposure), mice were sacrificed and lung homogenate was processed to monitor the *M. avium* and *M. tuberculosis* growth, as determined by CFUs. Results shown are the mean ± SEM for four mice per experimental group. These results indicate that mice exposed to silica and challenged with *M. avium* or *M. tuberculosis* resulted in a marked increase in *M. avium* or *M. tuberculosis* growth compared with control mice ($p < 0.01$ comparison of silica with either control dust or saline exposure). There were no significant differences in *M. avium* or *M. tuberculosis* growth between the two control groups (saline or Al_2O_3).

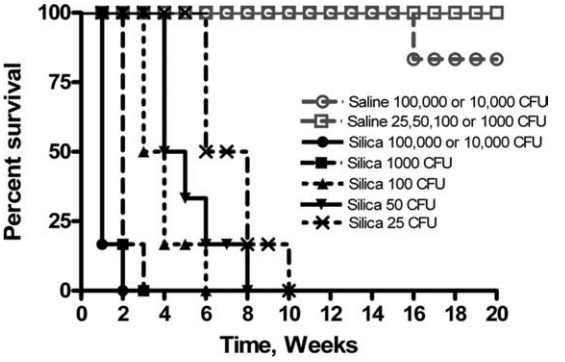

FIGURE 4. Survival of silica-exposed mice and control mice infected with *M. tuberculosis*. To determine the survival rate of the mice administered silica and challenged with *M. tuberculosis*, C57BL/6 mice were administered saline or silica I.T. After 30 days, all of the mice received the Erdman strain of *M. tuberculosis* (2.5×10^1–1×10^7) and mortality was determined over an 8-wk period ($n = 5$ mice/group). No deaths occurred in saline-treated control mice from any *M. tuberculosis* inoculum. The results are expressed as the mean ± SEM of three experiments performed in triplicate from four mice at each time point and are representative of two independent experiments. These results suggest that the *M. tuberculosis* Erdman strain is significantly more virulent in C57BL/6 mice treated with silica.

infection occurred during the same 1–2 wk following silica exposure associated with maximal cytotoxicity (Fig. 1*B*) and apoptosis (Fig. 1*C*) of AMs. In mice infected with *M. tuberculosis* at 3 wk after silica exposure, there was an increase in CFUs of *M. tuberculosis* at day 30 postinfection with a progressive increase in *M. tuberculosis* CFUs with initial infection from 4- to 16-wk postsilica exposure, similar to the results with *M. avium* (Fig. 2*B*). Consistent with previous results (41), *M. tuberculosis* infection of the control group (no silica) showed a reproducible organism burden of $\sim 6.5 \times 10^4$ CFUs at day 30 postinfection, which reflects a marked increase over the initial inoculum. Thus, *M. tuberculosis* growth remained stable in control C57BL/6 mice with a 10-fold increase in CFU numbers occurring in silica-exposed mice. These data demonstrate that silica exposure has a differential effect on susceptibility to *M. tuberculosis*, with resistance to infection early following silica exposure and enhanced susceptibility beyond 3 wk of exposure.

Susceptibility to mycobacteria infection is specific to silica exposure

We next confirmed that the increase in susceptibility to mycobacterial infection was specific to silica exposure and not simply a property of particulate exposure to the lung. C57BL/6 mice were administered saline, particulate Al_2O_3, or silica I.T. Thirty days later, all mice received *M. avium* (10^6) or *M. tuberculosis* (2.5×10^1). After an additional 30 days (60 days from particulate or saline exposure), the mice were sacrificed and mycobacteria in lung homogenate were determined. Mice exposed to silica showed a significant increase in the amount of *M. avium* or *M. tuberculosis* in their lungs compared with mice given Al_2O_3 or saline ($p < 0.01$ comparison of silica with either Al_2O_3 dust or saline exposure; Fig. 3). There was no significant difference in *M. avium*/*M. tuberculosis* CFUs between mice that received saline or particulate Al_2O_3 (Fig. 3, *A* and *B*, respectively).

Concentration-dependent mycobacterial infection in silica-exposed mice

Having established 30 days postsilica exposure as an optimal period for demonstrating increased susceptibility to mycobacterial infection, we sought to establish the threshold of mycobacterial exposure needed to establish infection. C57BL/6 mice were exposed to silica or saline. Thirty days later, they received increasing concentrations of *M. tuberculosis* ($2.5 \times 10^1 - 1 \times 10^6$). Since *M. tuberculosis* is more virulent than *M. avium*, the mice were observed every day for mortality after *M. tuberculosis* administration. Mice remaining alive at the end of the fourth week were sacrificed, lungs were homogenized, and *M. tuberculosis* growth was determined by CFUs. Silica-exposed mice demonstrated a concentration dependency with increased *M. tuberculosis* CFUs compared with saline control mice (data not shown). Silica-exposed mice that received *M. tuberculosis* between 10^5 and 10^6 did not survive beyond 1 wk. The silica-exposed mice that received 10^3 or 10^4 *M. tuberculosis* survived between 3 and 4 wk. However, mice that received 2.5×10^1–5×10^3 survived beyond 4 wk (Fig.

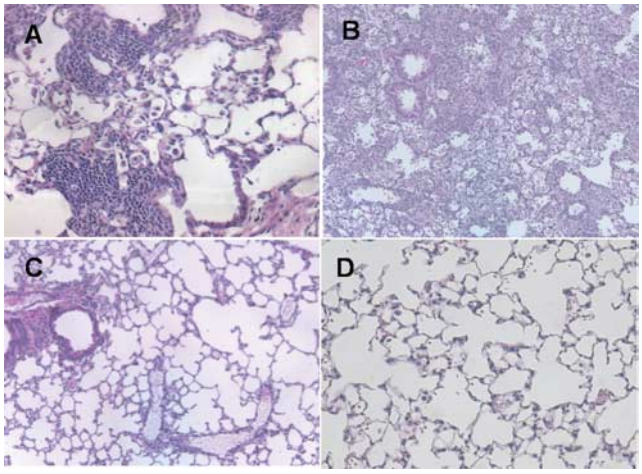

FIGURE 5. Morphologic evidence of silica-induced lung injury and increased mycobacterial infection. Silica-exposed mice revealed marked inflammatory cell infiltration at day 30 in the absence of *M. tuberculosis* infection (*A*). The cells were mixtures of macrophages, lymphocytes, and monocytes (data not shown). Further increases in cellular infiltration were noted in the lungs of *M. tuberculosis*-infected mice exposed to silica by day 60 (*B*). Lung sections from saline-exposed control animals in the presence (*C*) or absence (*D*) of *M. tuberculosis* infection did not exhibit any pathologic changes.

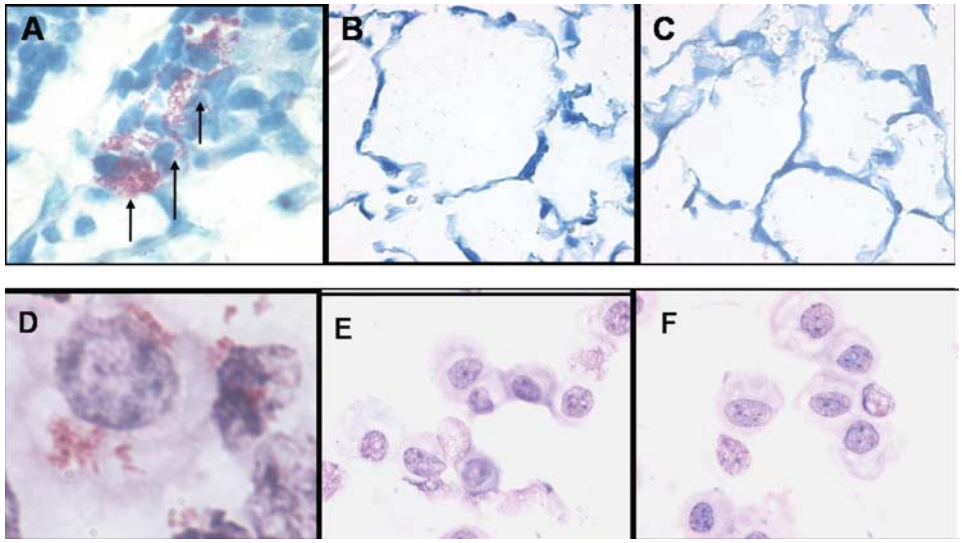

FIGURE 6. Increased *M. avium* in the lungs and AMs of silica-treated mice. Lung sections from silica-exposed mice reveal large numbers of AFB^+ bacilli as indicated by arrows in *A*. In contrast, there was no evidence of AFB^+ bacilli in the lungs of either Al_2O_3-treated mice (*B*) or saline-treated mice (*C*). Similarly, BAL cytopreparation smears from silica-exposed mice revealed large numbers of AFB^+ bacilli as indicated by arrows in *D* (original magnification, ×100). In contrast, no AFB could be detected in AMs of either Al_2O_3-treated mice (*E*) or saline-treated mice (*F*). These data indicate that silica-exposed mice have a marked increase in *M. avium* number compared with the control mice.

4). After 16 wk, only one death occurred in mice receiving saline instead of silica regardless of the concentration of the *M. tuberculosis* inoculum. Thus, the *M. tuberculosis* (Erdman) strain is significantly more virulent in C57BL/6 mice treated with silica. The optimal concentration of *M. tuberculosis* for our animal model was found to be 2.5×10^1 in silica-exposed mice and this concentration was used for the rest of the *M. tuberculosis* experiments.

Concentrations of *M. avium* at 10^3 resulted in low-level infection and concentrations of 10^8 resulted in high-level infection in silica-exposed mice. The concentration of 10^6 *M. avium* reproducibly established consistent growth during the 30 days of observation. The number of *M. avium* CFUs recovered from control C57BL/6 mice (no silica) increased only slightly with the increasing size of the *M. avium* inocula (10^3 CFU vs 10^8 CFU; data not shown).

Histopathology of silica and mycobacterial infection

To assess the histopathologic changes in the lung following silica exposure and *M. tuberculosis* infection, lung sections were stained by standard H&E and examined under light microscopy (Fig. 5). In response to silica exposure alone (i.e., before mycobacterial infection), lung tissue had evidence of monocytes, lymphocytes, and neutrophil infiltrations at day 30 (Fig. 5*A*). Further increases in inflammation were noted in the lungs of silica-exposed mice 30 days after they were infected with *M. tuberculosis* (Fig. 5*B*). There was moderate pathologic change in lung sections from saline-exposed mice after *M. tuberculosis* infection (Fig. 5*C*). Lung sections from saline-exposed control animals and uninfected mice did not exhibit any pathologic changes, regardless of whether or not they were subsequently infected with *M. tuberculosis* (Fig. 5*D*). Similar histopathologic changes were observed with *M. avium* (data not shown).

Consistent with the CFU determinations, the lung sections from silica-exposed mice showed an increased number of *M. avium*$^+$ bacilli as indicated with black arrows in Fig. 6*A*. In contrast, no acid fast bacilli (AFB) could be detected in either saline-treated mice (Fig. 6*B*) or in Al_2O_3-treated mouse lung sections (Fig. 6*C*).

To determine whether AMs from silica-exposed mice showed increase susceptibility to mycobacterial infection in vivo, C57BL/6 mice received silica, Al_2O_3, or saline. Thirty days after exposure, all mice were inoculated with *M. avium* (10^6; 60 days from silica exposure), mice were sacrificed, and AMs were isolated from the BAL. AMs from silica-exposed mice contained greater numbers of *M. avium* compared with mice exposed to Al_2O_3 or saline (Fig. 6, *D–F*).

To confirm that *M. tuberculosis* numbers were increased in silica-treated mice compared with control mice, C57BL/6 mice received I.T. silica or saline and after 30 days all of the mice received enhanced GFP (EGFP)-labeled H37Rv *M. tuberculosis* (2.5×10^1). Thirty days later, the mice were sacrificed. The confocal photomicrographs indicate that the number of EGFP-labeled *M. tuberculosis* in the lung sections of silica-exposed mice were significantly increased (Fig. 7*A*) compared with either saline-treated mice (Fig. 7*B*) or in Al_2O_3-treated mice lung sections (Fig. 7*C*). These data provide further evidence for the large number of EGFP-labeled *M. tuberculosis* present in AMs, suggesting that

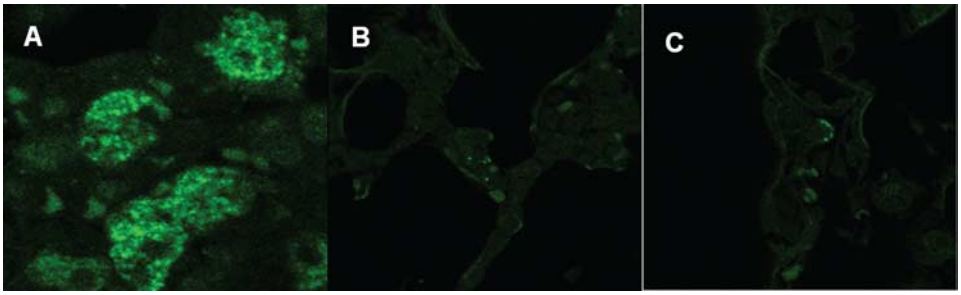

FIGURE 7. Increased *M. tuberculosis* in the lungs of silica-treated mice. Lung sections from mice exposed to silica and saline 4 wk before I.T. administration of EGFP *M. tuberculosis* were obtained after 4 wk of infection. The lung sections were observed under confocal microscopy for the presence of EGFP *M. tuberculosis* (original magnification, ×63). Lung sections from silica-exposed mice revealed large numbers of EGFP-labeled *M. tuberculosis* (*A*). In contrast, there were fewer numbers of EGFP bacilli present in the lungs of either Al_2O_3-treated mice (*B*) or saline-treated mice (*C*). These data indicate that silica-exposed mice have a marked increase in *M. tuberculosis* number compared with the control mice.

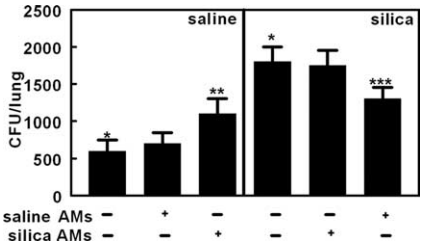

FIGURE 8. Passive transfer of AMs from silica-exposed mice to control mice increases *M. tuberculosis* susceptibility. Silica-exposed (4 wk post-exposure) or control C57BL/6 mice were administered I.T. AMs were harvested from silica-exposed mice or normal mice at a concentration of $0.5 \times 10^6/50\ \mu$l. All mice then received *M. tuberculosis* I.T. Thirty days later, the mice were sacrificed, lungs were removed, and the growth of *M. tuberculosis* from the lung homogenate was assessed by CFUs (*, $p < 0.05$ saline mice alone vs silica AMs (**, $p < 0.05$, control mice with *M. tuberculosis*) (*, $p < 0.05$ silica mice alone vs control AMs (***, $p < 0.05$, silica mice with *M. tuberculosis*). The results are expressed as the mean ± SEM of three experiments performed in triplicate and are representative of two independent experiments. These data suggest that passive transfer of silica-exposed AMs from silica-exposed mice to normal mice enhances in vivo susceptibility of mycobacterial infection.

AMs are the reservoir for the mycobacterial bacilli in the lung following silica exposure.

Passive transfer of AMs from silica-exposed mice to control mice increases M. tuberculosis *susceptibility*

To further assess the role of AMs in increasing susceptibility to mycobacterial infection following silica exposure, the lungs of C57BL/6 control mice were instilled with AMs harvested from silica-exposed mice or normal control mice at a concentration of $0.5 \times 10^6/50\ \mu$l. Groups of mice exposed to silica 4 wk before also received AMs harvested from either control or silica-exposed mice. All sets of mice then received I.T. *M. tuberculosis*. Thirty days after transfer of AMs and initiation of *M. tuberculosis* infection, the mice were sacrificed. Their lungs were removed and the number *M. tuberculosis* in the lung homogenate was assessed by CFUs. Administration of AMs from silica-exposed mice to control mice significantly increased *M. tuberculosis* growth in the normal mice (Fig. 8). Transfer of normal AMs into silica-exposed mice diminished *M. tuberculosis* growth (Fig. 8). Transfer of silica-exposed AMs into silica-exposed C57BL/6 mice showed no effect on *M. tuberculosis* levels (Fig. 8). Likewise, airway delivery of normal C57BL/6 AMs into recipient C57BL/6 mice resulted in similar low *M. tuberculosis* levels compared with normal C57BL/6 mice (Fig. 8). These data suggest that passive transfer of AMs from silica-exposed mice confers in vivo susceptibility to mycobacterial infection to normal C57BL/6 mice.

Discussion

Animal models remain an important tool in defining the pathogenesis of human infectious diseases. However, despite strong epidemiologic links between exposure to silica and susceptibility to mycobacterial infection in humans (4–6, 27–29), there have been no animal models described to our knowledge that confirm this association and can be used to elucidate the underlying mechanism(s) responsible. The current work is the first study showing that silica exposure markedly increases susceptibility to both *M. avium* and *M. tuberculosis* infection in mice. Furthermore, susceptibility to infection is associated with the appearance of repopulating AMs in the lungs following silica exposure.

The timing of the exposure to silica appears critical to the increased susceptibility to both *M. avium* and *M. tuberculosis*. No increase in mycobacterial susceptibility was seen during the initial 3 wk following silica exposure. In fact, in the first 2 wk following in vivo silica exposure, susceptibility to *M. tuberculosis* infection decreased relative to control mice. These results were surprising in view of prior in vitro studies reporting numerous deleterious effects on AM function following acute exposure to silica (19–25, 29), suggesting that silica-mediated AM injury might impair host defense and increase susceptibility to infection. However, the findings are consistent with previous AM depletion studies that demonstrate resistance to mycobacterial susceptibility (42).

The interaction of mycobacteria with AMs is complex. Ingestion of mycobacteria by AMs is a critical step in the control of mycobacterial infections. Following inhalation in an immune-competent host, mycobacteria are phagocytosed and killed by AMs. However, in situations where this initial response fails, viable bacilli use AMs as their primary site of replication, surviving and multiplying in a cellular environment protected from elimination by other components of the immune system (10, 14, 42–44).

Somewhat paradoxically, AMs also appear to be critical to the development of mycobacterial infection. Depleting AMs in mice with chlodronate (dichloromethylene diphosphonate) diminished the severity of pulmonary tuberculosis (42). This is not surprising since mycobacteria are intracellular pathogens that use macrophages as a "safe harbor" to grow and replicate (14). The presence of an adequate supply of healthy AMs may be critical for the establishment and maintenance of lung infection by *M. avium* and *M. tuberculosis* (42). Apoptosis of AMs has been suggested as a host defense mechanism against mycobacteria (45–49). In our studies *M. avium* or *M. tuberculosis* associated with AMs were dramatically increased in silica-exposed mice compared with mice exposed with control dust or saline, but an increased number of dead cells was also present. Silica-induced apoptosis increased in AMs over the first 2 wk and then declined. The percentage of apoptotic cells was returning to control levels at the time of increased susceptibility to *M. tuberculosis* infection (Fig. 2*C*). Perhaps the loss of healthy AMs early after silica exposure decreases susceptibility to *M. tuberculosis* (Fig. 2*B*) by decreasing the number of host cells capable of sustaining mycobacterial infection. AM recovery, presumably with repopulating viable AMs weeks later, may result in increased susceptibility (Fig. 2*B*).

AMs from silicotic mice appear to have a reduced ability to kill mycobacteria. Silica crystals in vitro adversely affect AMs as preincubating AMs with "sublethal" concentrations of quartz crystals results in a reduced ability to kill tubercle bacilli (22, 30). Our studies reveal that AMs from silica-exposed mice have more AFB^+ organisms than AMs from mice exposed to Al_2O_3 or control mice with *M. avium* alone. These infected AMs are present at the same time as AMs are repopulating the lungs following silica injury. It is not clear whether there is a specific subpopulation within these repopulating AMs that are responsible for the increased bacilli survival and replication.

Silica also affects susceptibility to other infections that are delayed by several weeks following exposure as we demonstrated for mycobacteria in this study. For example, mice are not susceptible to *Histoplasma capsulatum* 3–7 days after exposure to silica (50). However, if the time between silica exposure of mice and inoculation with *H. capsulatum* is increased to 14 or 21 days, enhanced susceptibility to infection occurs (50). In another study, rats exposed to silica for short time periods were not susceptible to *Listeria monocytogenes* infection (51). In contrast, silica exposure for almost 2 years increased susceptibility to *L. monocytogenes* (51, 52). These studies support our findings and suggest that a delayed effect following silica exposure may play a critical role in increasing susceptibility to a variety of intracellular pulmonary pathogens.

A key finding from our study is that AMs harvested from silica-exposed mice at a time when the mice exhibit increased susceptibility to mycobacterial infection transferred that increased susceptibility to mycobacteria to otherwise resistant control mice. Passive transfer of AMs from unexposed mice into silica-exposed mice significantly reduced the number of *M. tuberculosis* organisms. These results clearly indicate that AMs can transfer susceptibility to *M. tuberculosis*, suggesting that AMs are pivotal for silica-induced susceptibility. The mechanisms underlying macrophage programming that might induce mycobacterial susceptibility or resistance have yet to be fully elucidated.

Our data support the concept that changes in the alveolar environment after silica exposure and specifically a new population of macrophages may be linked to the increase in mycobacterial susceptibility following silica exposure. Immunologic properties of macrophages vary following defined activation stimuli (53). For example, the classically activated macrophages (M1) induced by Th1 signals (IFN-γ, TNF-α, and LPS) protect against intracellular pathogens as a result of increased oxidative burst, NO release, cytotoxic activities, and the production of proinflammatory cytokines (54, 55). In contrast, M2 macrophages induced by Th2 cytokines (e.g., IL-4) are generally characterized by low production of proinflammatory cytokines within the lung (56, 57). Previous studies suggest that macrophages from individuals with chronic inflammation resemble alternatively activated macrophages (M2) (58, 59). Although the presence of M2 macrophages has been linked to fibrosis, the presence of these macrophages appeared to diminish tissue injury and fibrosis following I.T. instillation of silica (56). Further studies will be needed to define the cellular and molecular markers of the population of AMs that appear 3 wk after silica exposure and are responsible for the enhanced susceptibility to mycobacterial infection.

Both macrophage-activating and -inactivating cytokines are produced following exposure to mycobacteria (60). However, whether local production of cytokines that specially impair AM function during the progression of silicosis contributes to susceptibility to mycobacterial infection is not known. The Th1 response is essential in protecting against *M. tuberculosis* infection; however, a Th2 cytokine profile subsequently results in increased susceptibility to *M. tuberculosis* and other opportunistic infections (60–65).

Previous studies suggest that the progression of silicosis is associated with a Th1 to Th2 switch in cytokines secretion (62). Acute silica exposure is associated with the induction of Th1 cytokines such as IL-2, IFN-γ, IL-12, and IL-18 (62, 66–73). However, other reports also suggest increases in Th2-type cytokines such as IL-4, IL-5, IL-9, IL-10, and IL-13 in silicosis (74). Preliminary studies from our laboratory have not identified any dramatic difference in the cytokine profile in the BAL of silica exposed relative to control mice at the time that we see enhanced susceptibility to mycobacterial infection (4 wk; data not shown). Nevertheless, it remains possible that a decrease in key macrophage-activating cytokines or enhancement in the levels of inhibitory cytokines in a later stage of silicosis could lead to the enhanced susceptibility to *M. avium* or *M. tuberculosis* disease due to impairment in AM immune functions.

In summary, we have demonstrated that silica pre-exposure for 30 days or more converts mycobacterial-resistant C57BL/6 mice into mice that are highly susceptible to both *M. avium* and *M. tuberculosis* infection. AMs from silica-exposed mice revealed higher numbers of AFB^+ *M. avium* compared with mice exposed to control dust or saline. Similarly, only lung sections from silica-exposed mice markedly increased *M. avium* and *M. tuberculosis* infection compared with control mice. In addition, passive transfer of silica-exposed AMs enhanced in vivo susceptibility of *M. tuberculosis* infection in normal C57BL/6 hosts. Direct or indirect effects of silica in the airway on AM populations appear to be critical in enhancing susceptibility to mycobacterial infection. The murine model that we describe herein offers an important new opportunity to uncover the link between human silicosis and mycobacterial infections of the lung.

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Disclosures

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