Decreased Asbestos-Induced Lung Inflammation and Fibrosis after Radiation and Bone Marrow Transplant

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The effect of lung irradiation on subsequent inflammatory or fibrotic lung injuries remains poorly understood. We postulated that irradiation and bone marrow transplantation might impact the development and progression of lung remodeling resulting from asbestos inhalation. Our objective was to determine whether irradiation and bone marrow transplantation affected inflammation and fibrosis associated with inhaled asbestos exposure. Inflammation, cytokine production, and fibrosis were assessed in lungs of naïve and sex-mismatched chimeric mice exposed to asbestos for 3, 9, or 40 days. Potential engraftment of donor-derived cells in recipient lungs was examined by fluorescence *in situ* hybridization and immunohistochemistry. Compared with asbestos-exposed naïve (nonchimeric) mice, chimeric mice exposed to asbestos for 3, 9, or 40 days demonstrated significant abrogation of acute increases in asbestos-associated inflammatory mediators and fibrosis. Donor-derived cells trafficked to lung but did not significantly engraft as phenotypic lung cells. Irradiation and bone marrow transplantation alters inflammatory and fibrotic responses to asbestos, likely through modulation of soluble inflammatory mediators.

Keywords: irradiation; asbestosis; stem cell; inflammation; fibrosis

Lung irradiation is used both for treatment of thoracic malignancies, including lung cancer, as well as part of total body irradiation in myeloablative regimens and subsequent bone marrow transplantation for hematologic and other malignancies. However, radiation can produce acute or chronic lung injury depending on dose rate, duration, pre-existing lung disease, concomitant corticosteroid use, and other factors (1–4). After nonmyeloablative radiation, acute radiation injury typically occurs 2 weeks to 3 months after treatment and is usually limited to the irradiated field. Mild injury often resolves without treatment, whereas more serious injury can result in fibrosis 6 to 12 months after treatment (1, 2). A number of mechanisms of damage by radiation in the lung, including oxidative stress, epithelial cell apoptosis, and other factors, have been reported (1–4). After myeloablative irradiation and bone marrow transplantation, additional donor-derived T lymphocyte-mediated pathologies, including idiopathic pneumonia syndrome (IPS), can occur (3, 4). In addition, recent evidence suggests that adult bone marrow-derived stem and/or progenitor cells can differentiate into structural cells, including fibroblasts (5), and that these cells can migrate to the

CLINICAL RELEVANCE

This article presents novel data that asbestos-induced lung inflammation and fibrosis are modulated following myeloablation and bone marrow transplantation.

lungs in response to fibrogenic stimuli, including lung irradiation (6–13). Although some studies have suggested amelioration of fibrotic lung injury by marrow-derived cells, a pro-fibrotic role for marrow-derived cells appears to occur in radiation-induced and other fibrotic lung injury models in mice (7–13).

However, despite a substantial literature on radiation-induced lung injury, less information is available concerning inflammatory and fibrotic responses to subsequent lung injury in previously irradiated lungs. This is an important consideration for patients who have undergone therapeutic lung irradiation, and would shed further light on how radiation alters inflammatory pathways in the lung. We were particularly interested in whether bone marrow-derived stem cells might play a role in subsequent acute or chronic lung injury after lung irradiation and speculated that marrow-derived cells might be protective in this regard. To evaluate this, we used a well-characterized model of asbestos-induced acute inflammation and subsequent fibrosis (14, 15) in sex-mismatched chimeric mice created by total body myeloablative radiation and subsequent bone marrow transplantation. Notably, we found that asbestos-induced inflammation and fibrosis was reduced in chimeric versus naïve mice. Although repopulation of the lung with adult marrow-derived cells occurred, only a small proportion of marrow-derived cells acquired a lung-specific immunophenotype. This suggests that the observed effects are not likely due to structural remodeling by marrow-derived stem cells but rather a modulation of asbestos-induced lung inflammation and fibrogenesis after irradiation and transplantation.

MATERIALS AND METHODS

All studies were subject to IACUC review at the University of Vermont (UVM) and conformed to institutional and American Association for Accreditation of Laboratory Animal Care (AAALAC) standards for humane treatment of laboratory animals. Before and after asbestos or sham exposure, mice were housed in pathogen-free barrier facilities in the Small Animal Facility at UVM.

Study Design and Exposures to Asbestos

Sex-mismatched chimeric adult (8–12 wk old) female C57Bl/6 mice or naïve female controls underwent 3-, 9-, or 40-day periods of exposure to asbestos (NIEHS reference sample of chrysotile asbestos at approximately 7 mg/m³ air) or clean air (sham groups) (Figure 1). Asbestos fiber characterization and size ranges have been reported previously (16). All asbestos and sham exposures were carried out for 6 hours/day, 5 days/week in chambers in the Animal Inhalation Facility at UVM, and sham animals were put in chambers with clean air (15, 16). Animals were offered food and water *ad libitum* during the exposure period. Three- and nine-day exposures were carried out in duplicate experiments.

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Figure 1. Experimental protocols for generation of chimeric mice and 3-, 9-, and 40-day asbestos exposures; $n = 5$ to 10 mice for each experimental group at each time point. Three- and nine-day exposures were carried out in duplicate.

Harvest, Expansion, and Administration of Total Bone Marrow Cells

Whole marrow was obtained from donor adult male (8–12 wk old) transgenic mice constitutively expressing green fluorescent protein (GFP) under the control of the ubiquitin promoter (courtesy of Philippa Marrack, Ph.D., National Jewish Medical Center, Denver, CO), washed, filtered to remove bone fragments and debris, and administered to adult female C57Bl6 recipient mice ($30\text{--}32 \times 10^6$ cells/mouse) by tail vein injection 4 hours after total body irradiation (1,000 cGy administered in a single dose) using a Cesium-137 cell irradiator (ISO 1000, Model B; Nordion International, Vancouver, BC, Canada) as previously described (17, 18). Engraftment was assessed 14 days after transplantation by determining the percent of peripheral leukocytes expressing GFP using flow cytometry. Mice were exposed to asbestos or clean air (sham groups) 30 days after cell administration.

Lung Analyses

Mice were killed at the indicated times (Figure 1), the lungs lavaged *in situ*, and the bronchoalveolar lavage fluid (BALF) assessed for cell counts and differential cell types as previously described (15, 16, 19). Additional aliquots of BALF were assayed for multiple chemokines and cytokines using a quantitative, fluorimetric, bead-based, multiplex approach (Bioplex; Bio-Rad, Hercules, CA) (15). At 40 days, nonacidic (latent) and acidic (active) transforming growth factor (TGF)- β_1 were measured in BALF using commercially available enzyme-linked immunosorbent assay.

Lungs were inflation fixed (20 cm) for 2 hours with ice-cold 4% paraformaldehyde, paraffin embedded, and 5 μm sections stained with hematoxylin and eosin (H&E) or Masson's trichrome stain before sections were scored using an established grading system (20) for inflammation and collagen deposition, respectively, in a blinded fashion by a board-certified pathologist (K.J.B.). To assess the presence of donor-derived cells in lungs, 5- μm sections were used for dual Y-chromosome painting and cell type-specific immunofluorescence staining using antibodies directed against CD45, Clara cell secretory protein (CCSP), or surfactant protein propeptide C (pro-SPC) (17, 18, 21). Secondary antibodies and fluorescence microscopy were used as previously described (17, 18).

Statistical Analyses

All data are graphed as means $\pm$ SEM. Statistical significance was evaluated by ANOVA using the Student Neuman-Keul's procedure for adjustment of multiple pairwise comparisons between treatment groups or by using the nonparametric Kruskal Wallis, Wilcoxon, and Mann-Whitney tests (22). Values of $P < 0.05$ were considered statistically significant.

RESULTS

An Attenuated Acute Inflammatory Response in BALF and Lungs of Asbestos-Exposed Mice Occurred after Myeloablation and Reconstitution of Bone Marrow

To evaluate the effects of myeloablation and reconstitution of bone marrow on asbestos-induced lung injury, adult (8–12 wk)

female C57Bl6 mice were myeloablated by total body irradiation, transplanted with total bone marrow cells from adult male GFP mice, and allowed to engraft for 30 days before the initiation of asbestos exposures. At 14 days after myeloablation/reconstitution, mice were engrafted with an average of 87% of peripheral leukocytes of donor origin. As we used myeloablative irradiation and marrow rescue, an experimental group of mice that were irradiated but not transplanted could not be included.

Total cell counts in BALF were increased ($P < 0.05$) in sham chimeric mice compared with sham naïve mice at 40 days (Figure 2A). After 9 days of asbestos exposure, asbestos-exposed naïve mice exhibited a significant increase ($P \leq 0.05$) in total cell counts compared with sham naïve mice. In contrast, 9-day asbestos-exposed chimeric mice demonstrated significant ($P \leq 0.05$) decreases in total cell numbers when compared with sham-exposed chimeric mice and to asbestos-exposed naïve mice (Figure 2A). At 40 days, both naïve and chimeric asbestos-exposed mice exhibited decreased total cells compared with naïve and chimeric sham mice, respectively ($P \leq 0.05$).

Differential cell counts on the BALF samples showed that compared with sham naïve controls on the same day, eosinophils were elevated ($P < 0.05$), concomitant with decreases in the percentage of macrophages ($P < 0.05$), at 3 and 9 days in asbestos-exposed naïve mice (Figure 2B). Although the percentages were small in comparison, increased numbers of lymphocytes and neutrophils were also observed at 9 days in asbestos-exposed naïve mice, the time point of peak lung inflammation in this model (15, 19). Asbestos-exposed chimeric mice showed significantly increased neutrophils at 3 and 9 days, with concomitant decreases in the percentage of macrophages ($P < 0.05$), compared with sham-exposed chimeric mice. However, percentages of lymphocytes, eosinophils, and neutrophils were lower in asbestos-exposed chimeric mice compared with asbestos-exposed naïve mice at 9 days, and eosinophils were also lower at 3 days ($P < 0.05$). At 40 days, both naïve and chimeric asbestos-exposed mice had increased numbers of neutrophils, eosinophils, and lymphocytes compared with corresponding sham-exposed animals ($P < 0.05$).

A number of cytokines and chemokines have been implicated in asbestos-induced lung injury and fibrosis, including TGF- β , TNF- α , IL-1, IL-4, IL-6, IL-8, IL-13, macrophage inflammatory protein (MIP)-1 α , MIP-1 β , and monocyte chemotactic protein (MCP)-1 (14, 15, 23). To determine effects of total body irradiation and transplantation on chemokine/cytokine elaboration as well as modulation after asbestos exposures, levels were measured in BALF using Bioplex technology (15). For the purpose of presenting the most relevant BALF cytokine and chemokine results, only information demonstrating statistically significant differences between asbestos-exposed chimeric vs

Figure 2. Irradiation and total marrow transplantation alters bronchoalveolar lavage fluid (BALF) total and differential cell counts after asbestos exposure. Total BALF cell counts (A) and cell differentials (B) in naïve and chimeric mice after 3, 9, or 40 days of exposure to clean air (sham) or asbestos. Data are expressed as mean $\pm$ SEM. Asb, asbestos; Eos, eosinophils; Lymph, lymphocytes; Mac, macrophages; Neu, neutrophils. *Significantly different ($P \leq 0.05$) from corresponding sham group (naïve or chimeric) at same time point; +Significantly different ($P \leq 0.05$) from similarly exposed (sham or asbestos) naïve group at same time point. For simplicity, the sham differential data depicted is from the 3-day naïve and 3-day chimeric groups, respectively. There were no significant differences in the 3-day sham differentials compared with 9-day or 40-day sham differentials in either naïve or chimeric mice.

asbestos-exposed naïve mice are included in Figure 3. (Additional data are included in Figure E1 in the online supplement.) In comparison to corresponding groups of sham mice, 3 days of asbestos exposure resulted in significant increases ($P < 0.05$) in levels of IL-5, IL-6, KC, and granulocyte macrophage (GM)-colony-stimulating factor (CSF) in asbestos-exposed naïve and chimeric mice (Figure 3). Significant increases in IL-1 β , IL-4, and MCP-1 were observed in asbestos-exposed naïve mice, but not in asbestos-exposed chimeric mice ($P < 0.05$). IL-13 was significantly increased in asbestos-exposed naïve mice and significantly decreased ($P < 0.05$) in asbestos-exposed chimeric mice compared with corresponding sham controls. MIP-1 α was also decreased in asbestos-exposed chimeric mice compared with corresponding sham chimera ($P < 0.05$).

At 9 days, asbestos exposures resulted in significant increases in IL-1 β , IL-4, IL-5, IL-6, IL-12p40, MIP-1B, KC, granulocyte-CSF (G-CSF), and MCP in both naïve and chimeric mice compared with the corresponding sham-exposed group ($P <$

0.05, Figure 3). However, for IL-4, IL-5, IL-12p70, MIP-1 β , and MCP-1, the response in asbestos-exposed chimeric mice was significantly less than in asbestos-exposed naïve mice ($P < 0.05$). Asbestos also induced increases in RANTES in asbestos-exposed naïve mice but not in asbestos-exposed chimeric mice ($P < 0.05$). When sham chimeric mice were compared to sham naïve mice, a significant increase in IL-12p40 was accompanied by significant decreases in IL-1 α , IL-4, IL-12p70, and MIP-1 β ($P < 0.05$, Figure 3).

At 40 days, IL-1 β , G-CSF, and KC were increased ($P < 0.05$) in asbestos-exposed chimeric mice, but not in asbestos-exposed naïve mice (Figure 3). Levels of IL-5, IL-6, IL-12p40, MIP-1 β , and MCP-1 were increased in both naïve and chimeric asbestos-exposed mice compared with corresponding sham-exposed mice, but levels of IL-6, IL-12p40, and MIP-1 β were significantly lower in asbestos-exposed naïve mice compared with asbestos-exposed chimeric mice ($P < 0.05$). Compared with sham naïve mice, sham chimeric mice showed a significant

Figure 3. Irradiation and total marrow transplantation alter BALF cytokine and chemokine levels after asbestos exposure. BALF was simultaneously analyzed for multiple cytokines and chemokines utilizing a multiplex (Bio-Rad) system. Values represent means $\pm$ SEM of 5 to 10 samples for each condition at each time point. *Significantly different ($P \leq 0.05$) from corresponding sham group (naive or chimeric) at same time point; +Significantly different ($P \leq 0.05$) from similarly exposed (sham or asbestos) naive group at same time point.

increase in G-CSF, and significantly decreased levels of IL-1 α , IL-12p70, IL-13, KC, MCP-1, and IFN- γ ($P < 0.05$). RANTES levels were higher in asbestos-exposed chimeric mice compared with both sham chimeric and asbestos-exposed naive mice ($P < 0.05$). No significant differences were observed between experimental groups at any time point for BAL fluid levels of IL-10, IL-17, or eotaxin. TNF- α levels were undetectable.

Levels of acidic TGF- β_1 were significantly increased in BALF of 9 and 40 day asbestos-exposed compared to sham-exposed naive mice ($P < 0.05$, Figure 4). However, there was a significant decrease in acidic TGF- β_1 in chimeric asbestos-exposed mice at 9 days that was not observed at 40 days ($P <$

0.05). Nonacidic TGF- β_1 levels were comparable in all groups (data not shown).

Donor-Derived Stromal Marrow Cells Localized in Lungs of Asbestos-Exposed Chimeric Mice

Large numbers of donor-derived (Y chromosome-positive) cells were identified in lungs of chimeric mice (Figures 5 and 6). The majority of these stained positively for CD45, suggesting leukocyte identity. In contrast to sham mice (Figures 5A and 5B), peribronchiolar accumulations of leukocytes were observed after exposure of chimeric mice to asbestos at 3 (Figure 5C) and 9 days (Figure 5E). At 40 days, peribronchiolar

Figure 4. Irradiation and total marrow transplantation decrease BALF acidic TGF- β_1 levels after 9 but not after 40 days of asbestos exposure. Values represent means $\pm$ SEM of 5 to 10 samples for each condition at each time point. *Significantly different ($P \leq 0.05$) from corresponding sham group (naïve or chimeric) at same time point; +Significantly different ($P \leq 0.05$) from similarly exposed (sham or asbestos) naïve group at same time point.

accumulation of leukocytes was noted in sham (Figures 6A and 6B) as well as asbestos-exposed animals (Figures 6C and 6D). In all groups, donor-derived cells within the airways stained positively for CD45, but did not exhibit positive staining for CCSP. However, a number of donor-derived cells localizing in the parenchyma did not stain positively for either CD45, CCSP, or pro-SPC. Based on this information, we speculate that these cells may have acquired interstitial phenotype. Table 1 shows the quantitation of donor-derived cell types in the lungs of sham and asbestos-exposed chimeric mice using fluorescence *in situ* hybridization (FISH) and immunohistochemistry (IHC). Notably, the percentage of donor-derived cells repopulating the lung increased over time in both sham and asbestos exposed mice. The average percentage of CD45⁺/Y⁺ cells ranged from 7.6 to 10% in lungs of sham mice and from 10.8 to 16.3% in lungs of asbestos exposed mice. At 3 and 40 days, significantly increased ($P < 0.05$) numbers of CD45⁺/Y⁺ cells were seen in the lungs of asbestos vs. sham mice (Table 1).

Lung Fibrosis Was Significantly Reduced in Chimeric Mice after 40 Days of Asbestos Exposure

In both naïve and chimeric mice, asbestos exposures for 40 days resulted in significant ($P \leq 0.05$) increases in fibrosis that was centered around the terminal bronchioles as determined using Masson's trichrome stain to detect collagen deposition (Figure 7). However, when compared with asbestos-exposed naïve mice, which exhibited both inflammation and increased peribronchiolar collagen deposition (Figure 7B), asbestos-exposed chimeric mice demonstrated significantly less inflammation and fibrosis (Figure 7, Table 2). Neither sham naïve nor sham chimeric animals demonstrated increased peribronchiolar fibrosis or inflammation. These latter data suggest that myeloablation and transplantation alone did not cause the inflammatory lung injury or fibrosis in this model.

A summary of the differences in inflammation and fibrosis between experimental groups is presented in Table 3.

DISCUSSION

The results of this study demonstrate that myeloablative irradiation followed by bone marrow transplantation altered

inflammatory and fibrotic lung injury resulting from subsequent inhalation of chrysotile asbestos fibers. As summarized in Table 3, myeloablation and transplantation resulted in diminution of several indicators of lung inflammation after acute asbestos exposures for 3 and 9 days. Importantly, after 40 days of asbestos inhalation, chimeric mice demonstrated markedly reduced lung fibrosis when compared to naïve mice comparably exposed to asbestos. These results are less likely due to engraftment of marrow-derived cells as epithelial or interstitial cells in chimeric mouse lungs but may rather reflect alterations in lung inflammatory and immune responses after irradiation and transplantation.

Rodent models of asbestos inhalation have proven useful for studying the inflammatory and subsequent fibrotic changes characteristic of clinical asbestosis. Exposure to asbestos at airborne concentrations comparable to those used in this study have resulted in prolonged inflammatory responses characterized by increased proportions of polymorphonuclear leukocytes (PMNs), both neutrophils and eosinophils, in BALF as well as inflammatory foci in terminal bronchiolar and alveolar duct regions of the lung (16, 23, 24). Transient proliferation of bronchiolar and alveolar type II epithelial cells have been observed within days after initial exposures to asbestos followed by subsequent proliferation of cells in the interstitial compartment of the lung (15, 16, 19, 23–28). The origin of the cell types involved in asbestos-induced epithelial proliferation and subsequent fibrosis has been presumed to be resident cells in the lung. However, a recent study has evaluated the role of bone marrow cells in asbestos-induced lung injury. In this study, sex-mismatched chimeric rats exposed to asbestos for 3 days demonstrated clusters of donor-derived cells in areas of bronchoalveolar duct bifurcations, the site of asbestos-induced fibrosis in this model (29). While initially the majority of cells were monocytes or macrophages, at later time points some exhibited immunohistochemical phenotype of myofibroblasts or fibroblasts and also some of the donor-derived cells appeared to be proliferating. This suggests that a population of bone marrow-derived cells, perhaps fibrocytes as has been demonstrated in other models of fibrosis (8–13), may play a pathogenic role in asbestos-induced fibrosis. In addition, rare type 2 alveolar epithelial cells that appeared to be donor-derived were identified.

In parallel, our studies suggest that asbestos treatment of chimeric mice only resulted in rare airway or alveolar epithelial cells that appear to have originated from marrow-derived cells, similar to what we have observed with lung injury induced by naphthalene, endotoxin, or NO₂ in chimeric mice (17, 18). The dose of radiation used in our studies was comparable to that used in other studies evaluating engraftment of lung epithelium by marrow-derived cells (30, 31). The small numbers of these cells makes it unlikely that they play a meaningful role in asbestos-induced structural remodeling. There were more numerous interstitial cells that appear to be bone marrow derived, and these were increased in asbestos versus sham exposed chimeric mice. These arguably might play a role in asbestos-induced lung injury and further characterization of these cells both as to identity and origin (i.e., derived from circulating fibrocytes or other cell populations) is required to better understand their role.

The role of specific inflammatory cells and of specific inflammatory cytokines and chemokines in development of asbestos-induced lung pathology is only partly understood (14, 15, 23). Alveolar macrophages play a prominent role in phagocytosing asbestos particles and mRNA levels of both TNF- α and IL-1 β are increased in alveolar macrophages obtained from patients with asbestosis (32), whereas alveolar thickening does not develop in TNF- α knockout mice exposed

Figure 5. Chimeric mice exposed to asbestos for 3 and 9 days demonstrate trafficking of donor-derived cells to the lungs. Donor-derived cells were revealed by fluorescence *in situ* hybridization (FISH) for the Y chromosome (red). (A) Donor-derived cells in interstitium (indicated by arrow). *Inset* in A is higher power view of the area indicated by arrow showing three donor-derived cells that are negative for the leukocyte marker CD45 (blue) and the lung-specific marker CCSP (green). (B) Y-positive donor-derived cell in interstitium (arrow) that is negative for CD45 but positive for pro-SPC (green). *Inset* in B is a higher power view of area indicated by arrow. (C) Donor-derived CD45-positive cells (blue) in airway, and (D) in interstitium. *Inset* in C is a higher power view of the area indicated by arrow showing peribronchiolar accumulation of donor-derived leukocytes (blue). (E) CCSP-positive airway epithelial cell (green) indicated by white arrow. *Inset* in E is a higher power view of the area indicated, revealing no donor-derived cells in epithelia. (F) pro-SPC-positive cells in lung interstitium (green), indicated by white arrows, are not donor derived. *Inset* in F is a higher power view of the indicated cell. Confocal immunofluorescence; original magnifications: A, C, and E, $\times 200$; B, D, and F, $\times 400$. Asb, asbestos.

to asbestos (28). Both of these, as well as other pro-inflammatory chemokines, including MIP1 α and MCP-1, are increased in lung and BALF in rodent models of asbestos exposure (14, 23). Importantly, these inflammatory mediators are increased soon after initial asbestos exposure, preceding the development of lung fibrosis, and highlight a prominent role of alveolar macrophages in asbestos-induced inflammation. The subsequent interactions of these agents and their relative roles in promoting fibrosis are complex, but available data suggest key pro-fibrotic roles of TNF- α and TGF- β (33).

The role of other inflammatory cells, including T lymphocytes and eosinophils, in asbestos-induced lung inflammation and fibrosis is less clear. We have recently demonstrated increased BALF levels of several pro-fibrotic Th2 cytokines, including IL-4, and the pleiotropic cytokine IL-6 after 9 days of asbestos exposure in mice (15). In the present work, levels of IL-4, IL-5, and IL-13, major effector molecules influencing Th2 inflammation and lung remodeling, as well as several IL-13-induced chemokines (MIP-1 β , RANTES), were significantly elevated after 3 and/or 9 days of asbestos exposure. While

eosinophils are also elevated in lungs of asbestos-exposed mice, their pathogenic role remains unclear (15).

Our current studies demonstrate that BALF eosinophils were significantly decreased 3 and 9 days and lymphocytes significantly decreased 9 days after asbestos exposure in chimeric mice. Furthermore, a number of asbestos-induced increases in acute pro-inflammatory cytokines and chemokines were abrogated in chimeric versus naïve mice. Notably, mediators of Th2 inflammation, including eosinophils and Th2 cytokines (including IL-4, IL-5, and IL-13), were particularly decreased at 3 and/or 9 days. Surprisingly, the transplantation procedure itself, including total body and thus lung irradiation, had relatively little effect on basal levels of inflammatory cells or of these acute inflammatory mediators measured in BALF as determined by comparing chimeric versus naïve groups not exposed to asbestos, although several other cytokines were decreased in sham-exposed chimeric versus naïve mice. Radiation pneumonitis and subsequent lung fibrosis in mice can occur with the radiation dose used in our study (7, 30, 31). However, the effects usually manifest later, both clinically and in experimental

Figure 6. Chimeric mice demonstrate trafficking of donor-derived cells to the lung and accumulation of peribronchiolar leukocytes at 40 days. Donor-derived cells were revealed by FISH for the Y chromosome. Leukocytes (blue) demonstrate positive staining for CD45. (A) Peribronchiolar accumulation of leukocytes in a sham lung is indicated by the arrow. Inset in A is higher power view of indicated area showing leukocytes and CCSP-positive cells (green) in airway epithelia. (B) Type II alveolar epithelial cells showing positive staining for proSPC (green) and leukocytes in lung interstitium of a sham animal. Inset in B is a higher power view of the area indicated by an arrow showing a marked accumulation of donor-derived cells in the airway epithelia. (C) Lung interstitium and airway of a mouse exposed to asbestos for 40 days showing positive staining for CCSP (green). The arrow indicates an accumulation of leukocytes in the interstitium. (D) pro-SPC-positive cells in lung interstitium of a mouse exposed to asbestos for 40 days (green). Inset in D is a higher power view of the area indicated by the arrow showing donor-derived leukocytes. Confocal immunofluorescence; original magnification: $\times 400$.

models, than the time course we employed for evaluation of acute asbestos effects in chimeric mice, 33 and 39 days, respectively, after myeloablation and transplantation (7, 34).

Nonetheless, despite relatively minimal effects on baseline inflammatory markers by irradiation alone, asbestos inhalation after irradiation revealed a diminished capacity of the lung to mount both acute inflammatory and subsequent fibrotic responses. Surprisingly, relatively little is known about the effect of lung irradiation on the subsequent inflammatory and immune behavior of the lung in response to subsequent challenges with inflammatory or fibrotic agents. Sublethal and lethal lung irradiation can trigger a number of genetic and molecular events that can have both short-term effects and longer-term effects, often following a clinically occult latency period. Some of the more immediate effects include recruitment of inflammatory cells to lung, up-regulation of adhesion molecules, induction of oxidative injury, and generation of a number of pro-inflammatory cytokines and chemokines, notably $\text{TNF-}\alpha$ (34–38). Studies of clinical bone marrow transplantation, involving lethal total body irradiation pre-conditioning, and parallel studies in mouse models of marrow transplantation, have further implicated donor-derived host-reactive T cells in subsequent lung injuries, including IPS (39–41). While also mediated in part by oxidative injury, a role for $\text{TNF-}\alpha$ and for endotoxin translocated across

the intestinal epithelium has been proposed in the pathogenesis of IPS (35–37). However, while providing much useful information on pathogenesis of radiation-induced pathologies in lung, these studies have not addressed the response of the irradiated lung to subsequent inflammatory or fibrotic stimuli.

TABLE 1. DONOR CELL ENGRAFTMENT IN LUNGS OF CHIMERIC MICE

Condition (N)	% Donor-Derived Cells/Total Cells	Normalized % Donor-Derived Cells/Total Cells*	% CD45-Cells/Y+ Cells
Sham, 3 d (2)	14.4 ($\pm$ 2.5)	21.1 ($\pm$ 3.6)	9.1 ($\pm$ 2.0)
Asbestos, 3 d (2)	19.0 ($\pm$ 1.6)	27.7 ($\pm$ 2.3)	13.8 ($\pm$ 0.94) [†]
Sham, 9 d (1)	23.9	35.0	7.0
Asbestos, 9 d (2)	19.0 ($\pm$ 1.9)	27.9 ($\pm$ 2.7)	10.8 ($\pm$ 0.56)
Sham, 40 d (2)	33.0 ($\pm$ 0.19)	48.6 ($\pm$ 0.45)	10.0 ($\pm$ 0.21)
Asbestos, 40 d (2)	33.6 ($\pm$ 2.3)	49.3 ($\pm$ 3.3)	16.3 ($\pm$ 0.22) [†]

Approximately 16,000 total cells were evaluated. Seven to 12 nonadjacent fields/lung (5 μm sections) were examined at $\times 40$ magnification.

* The detection of donor-derived cells was normalized to the fraction of cells showing Y positivity by FISH in male control lung sections (68.3%). Approximately 1,500 cells/lung were evaluated ($n = 4$) totaling approximately 6,000 cells.

[†] Significantly different from sham on the same day ($P \leq 0.05$).

Figure 7. Chimeric mice show a reduction in pulmonary fibrosis after 40 days of exposure to asbestos. Lung sections were stained with Masson’s trichrome to evaluate collagen deposition (dark blue). (A) Minimal collagen deposition surrounding distal airway of a naïve sham mouse. (B) Distal airway of naïve mouse exposed to asbestos demonstrating extensive peribronchiolar fibrosis (indicated by arrow). (C) Chimeric sham mouse showing no increase in peribronchiolar collagen deposition when compared with naïve sham mouse (A). (D) Distal airway of an asbestos-exposed chimeric mouse demonstrating minimal peribronchiolar collagen deposition. (E) Alveolar duct junction of an asbestos exposed chimeric mouse demonstrating minimal peribronchiolar collagen deposition. Original magnification: $\times 200$.

There is also little information about inflammatory cell turnover in lung after myeloablative irradiation and transplantation or whether inflammatory cells in lung, either donor-derived or residual host-derived, function normally after myeloablative irradiation and transplantation. In our study, we allowed the recipient mice a 30-day period for engraftment before asbestos exposure, a reasonable and frequently used interval affording good peripheral engraftment and with return of normal apparent functional capacity of engrafted neutrophils and lymphocytes to respond to inflammatory stimuli. However, alveolar macrophages may require up to 3 months for full turnover after irradiation and transplantation (42). Further, little is known about eosinophil function or contribution to lung inflammation or fibrosis following irradiation and transplantation. Our results, in which levels of BALF cytokines and chemokines were mostly comparable between sham-exposed naïve and chimeric mice, suggest that inflammatory cells, a significant source of many of the measured inflammatory mediators, had appropriate basal function at the early time points assessed in this study. How-

ever, the response to asbestos exposure was significantly altered in chimeric versus naïve mice. The mechanism(s) for these effects are unclear at present, and further research is warranted concerning the function of transplanted inflammatory cells, and

TABLE 2. ASSESSMENT OF FIBROSIS IN NAÏVE VERSUS CHIMERIC MICE AT 40 DAYS AFTER INITIATION OF EXPOSURE TO ASBESTOS

Group	Mice/Group	Severity*	Avg % Bronchioles Affected
Sham, naïve	6	1.0	0
Asbestos, naïve	8	2.9†	65.6
Sham, chimeric	5	1.0	0
Asbestos, chimeric	5	1.8‡§	27.0

* Grading of fibrosis: 1. no fibrosis; 2. mild fibrosis; 3. moderate fibrosis; 4. severe fibrosis.

† Significantly different from Sham, naïve, $P < 0.05$.

‡ Significantly different from Sham, chimeric, $P < 0.05$.

§ Significantly different from Asb 40 days, naïve, $P < 0.05$.

TABLE 3. SUMMARY OF RESULTS COMPARING CHIMERIC TO NAÏVE MICE

Sham Chimeric versus Sham Naïve				
Endpoint:	3 d	9 d	40 d	
Total Cell Counts	ND	ND	↑	
Differential Cell Count	ND	ND	ND	
BALF Cytokines and Chemokines	Increase	IL-13	None	IL-1 α G-CSF
	Decrease	None	IL-1 α IL-4 IL-12p40 IL-12p70 MIP-1 β	IL-12p70 MCP-1 KC IFN- γ None
Fibrosis	—	—	—	None
Asbestos Chimeric versus Asbestos Naïve				
Endpoint:	3 d	9 d	40 d	
Total Cell Counts	ND	ND	ND	
Differential Cell Count	↓ Eosinophils	↓ Eosinophils, Lymphocytes, Neutrophils	ND	
BALF Cytokines and Chemokines	Increase	↑ Macrophages None	↑ Macrophages None	ND IL-1 β IL-6 IL-12p40 MIP-1 β RANTES KC G-CSF
	Decrease	IL-4 IL-12p40 IL-13 MCP-1	IL-4 IL-5 IL-12p70 MIP-1 β MCP-1	IL-12p70
Fibrosis	—	—	Decreased	

Definition of abbreviations: BALF, bronchoalveolar lavage fluid; G-CSF, granulocyte colony-stimulating factor; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; ND, no difference; RANTES, regulated on activation, normal T cells expressed and secreted; —, not evaluated.

of residual irradiated host-derived inflammatory cells, as well as effects of irradiation on release of inflammatory mediators by lung epithelial cells, in subsequent inflammatory and fibrotic effects in the lung.

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

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