The aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor, is responsible for mediating a variety of pharmacological and toxicological effects caused by halogenated aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). However, recent evidence has revealed that the AhR also has numerous physiological roles aside from xenobiotic metabolism, including regulation of immune and inflammatory signaling as well as normal development and homeostasis of several organs. To investigate the role of the AhR in crystalline silica (SiO2)-induced inflammation and fibrosis, C57Bl/6 and AhR−/− mice were exposed to SiO2 or vehicle. Similarly, C57Bl/6 mice were exposed to SiO2 and TCDD either simultaneously or sequentially to assess whether AhR activation alters inflammation and fibrosis. SiO2-induced acute lung inflammation was more severe in AhR−/− mice; however, the fibrotic response of AhR−/− mice was attenuated compared with C57Bl/6 mice. In a model of chronic SiO2 exposure, AhR activation by TCDD in C57Bl/6 mice resulted in reduced inflammation; however, the fibrotic response was not affected. Bone marrow–derived macrophages (BMM) from AhR−/− mice also produced higher levels of cytokines and chemokines in response to SiO2. Analysis of gene expression revealed that BMM derived from AhR−/− mice exhibit increased levels of pro-interleukin (IL)-1β, IL-6, and Bel-2, yet decreased levels of signal transducers and activators of transcription (STAT)2, STAT5a, and serpin B2 (Pai-2) in response to SiO2.

Key Words: lung; inflammasome; serpin B2 (Pai-2); interleukin 1β.
AhR regulates silica-induced inflammation

Although models focused on early events in silicosis have yielded insight into underlying mechanisms such as the Nlrp3 inflammasome that initiate injury; little is known about mechanisms that resolve inflammation. The aryl hydrocarbon receptor (AhR) plays a substantial role in the immune system and is best known as the receptor for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Activation of the AhR by TCDD results in a range of toxic endpoints including profound immunosuppression (Lawrence and Kerkvliet, 2007; Marshall and Kerkvliet, 2010). Moreover, several natural or endogenous ligands bind to the AhR with variable affinity and potency (Thomas et al., 2002). These compounds contain tryptophan derivatives, such as 6-formylindolo[3,2-b]carbazole, IAA (indole-3-acetic acid), tryptamine, indirubin, ICZ (indolo[3,2-b]carbazole), and DIM (3,3’-diindolylmethane), prostaglandins, such as PGG2 and lipoxin A4, and heme metabolites, including bilirubin and biliverdin (Fuji-Kuriyama and Kawajiri, 2010). A majority of the cells that participate in immune responses constitutively or inducibly express the AhR (Fuji-Kuriyama and Kawajiri, 2010; Kerkvliet, 2009). Many genes that participate in immune responses have dioxin response element sequences in their promoters and are responsive to AhR ligands (Kerkvliet, 2009; Stevens et al., 2009). The effects of TCDD and presumably AhR activation on inflammatory responses have been studied for many years with much conflicting data in the literature. Early studies reported that exposure to TCDD greatly enhanced lipopolysaccharide (LPS) toxicity (Vos et al., 1978); yet AhR$^{-/-}$ mice are hypersensitive to LPS-induced inflammatory responses, including IL-1β production (Kimura et al., 2009; Sekine et al., 2009). Consistent with these in vivo results, Kimura et al. revealed that peritoneal macrophages isolated from AhR$^{-/-}$ mice secreted higher levels of proinflammatory cytokines than those from wild-type mice in response to LPS. Furthermore, transfecting AhR$^{-/-}$ macrophages with an AhR expression plasmid suppressed this response (Kimura et al., 2009; Sekine et al., 2009). Together, these results indicate that the AhR suppresses the expression of inflammatory cytokines in response to LPS. With regard to inflammasome activation and IL-1β secretion in particular, the AhR activates the expression of serpin b2/Pai-2 (Sekine et al., 2009), an inhibitor of caspase-1 activation (Greten et al., 2007). AhR ligands augment Pai-2 expression in wild-type macrophages, and transduction of Pai-2 into AhR$^{-/-}$ macrophages restored suppression of IL-1β secretion (Sekine et al., 2009). Activated AhR together with nuclear factor-kappa B (NF-kB), but not with Amt, directly enhanced the expression of Pai-2 gene expression by binding to its promoter (Sekine et al., 2009). Therefore, a better understanding of Mø activation and regulation of inflammatory signaling by the AhR may provide insights into silicosis and may serve as potential targets for therapeutic intervention. In this study, we demonstrate that AhR$^{-/-}$ mice are more sensitive than C57Bl/6 mice, to SiO$_2$-induced inflammation, but not fibrosis. Similarly, AhR activation by TCDD in C57Bl/6 mice reduced inflammation but had no effect on the development of fibrosis. Macrophages derived from AhR$^{-/-}$ mice secreted enhanced amounts of cytokines and chemokines in response to SiO$_2$. Analysis of gene expression revealed that AhR$^{-/-}$ macrophages exhibit increased levels of pro-IL-1β, IL-6, and Bcl-2, yet decreased levels of signal transducers and activators of transcription (STAT)2, STAT5a, and serpin B2 (Pai-2) in response to SiO$_2$. Finally, activation of the inflammasome following stimulation of bone marrow-derived macrophages (BMM) with SiO$_2$ plus LPS for 24 h resulted in elevated levels of IL-1β present in tissue culture supernatants. This response was reduced by concomitant treatment with 10nM TCDD in wild-type macrophages. In contrast, no change was observed in the levels of IL-1β present in tissue culture supernatants in BMM derived from AhR$^{-/-}$ mice.

**MATERIALS AND METHODS**

**Mice.** Breeding pairs of C57Bl/6 (C57BL/6J, stock #000664) mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME). AhR$^{-/-}$ (B6.AhR$^m1{Boy}$) mice were obtained from Dr Paige Lawrence (University of Rochester Medical College, Rochester, NY) and bred as previously described (Schmidt and Bradfield, 1996). All mice were maintained in the University of Montana specific pathogen free facility and used at 6–8 weeks of age. All animal use procedures were in accordance with National Institutes of Health and University of Montana Institutional Animal Care and Use Committee guidelines.

**Experimental instillations.** Crystalline silica (SiO$_2$, 1.5–2 μm) (Pennsylvania Glass Sand Corporation, Pittsburgh, PA) was acid washed, dried, and determined to be free of endotoxin (data not shown). Mice were anesthetized with isoflurane and instilled via the oropharyngeal exposure route with 25 μl sterile saline (vehicle) or 1 mg SiO$_2$ suspended in 25 μl of sterile saline. For acute exposure studies, tissues were collected and assayed at 1, 3, 7, and 33 days following the initial instillation. For chronic exposure studies, mice were anesthetized with isoflurane and exposed to 25 μl vehicle or 1 mg SiO$_2$ suspended in 25 μl of vehicle once a week for 4 weeks prior to study termination on day 28. In the sequential exposure paradigm, mice were anesthetized with isoflurane and exposed to 25 μl vehicle or 1 mg SiO$_2$ suspended in 25 μl of vehicle once a week for 4 weeks prior to weekly vehicle or TCDD oral gavage for the next 8 weeks. In the concomitant exposure paradigm, mice were gavaged weekly with vehicle or TCDD (see below), beginning 1 day prior to being anesthetized with isoflurane and exposed to 25 μl vehicle or 1 mg SiO$_2$ suspended in 25 μl of vehicle (Supplementary fig. 1). Mice were then returned to their cages and monitored until mobility returned.

2,3,7,8-Tetrachlorodibenzo-p-dioxin. TCDD was obtained from Cambridge Isotope Laboratories Inc. (Woburn, MA). For in vivo experiments, TCDD was initially dissolved in anisole (Sigma-Aldrich, St Louis, MO) and further diluted in peanut oil. Mice were gavaged with 200 μl peanut oil (po, vehicle) or 10 μg/kg TCDD (suspended in vehicle), a dose previously established to be immunosuppressive in several mouse models of immune-mediated disease (Lawrence et al., 2006; Teske et al., 2005). TCDD used for in vitro cultures was dissolved in dimethyl sulfoxide (DMSO) and used at 10nM, an environmental and physiologic concentration.

**Assessment of pulmonary inflammation.** Mice were euthanized and whole lung lavage (WLL) performed by cannulating the trachea and infusing the lungs with 1 ml sterile 0.9% NaCl, EDTA/PBS. The acellular lavage fluid was collected by centrifugation and frozen at −20°C until further analysis. Total WLL cells were recovered and either: (1) spun onto cytospin slides, differential...
counts determined by two independent readers and expressed as absolute cell number or (2) prepared for multicolor flow cytometric analysis as described below. Lung tissue was homogenized with a Tissue-Tearor in 1 ml radioimmuno precipitator assay lysis buffer (10mM 4-2-hydroxyethyl)-1-piperazinethanesulfonic acid, 150mM NaCl, 1mM EDTA, 0.6% Nonidet P-40, and 5mM PMSF, with HALT protease inhibitor cocktail, according to the manufacturer’s instructions) on ice. After homogenization, the samples were centrifuged at 14,000 × g for 30 min and the clarified supernatants stored at −20°C until assayed (Beamer et al., 2010). Additional lung tissue was dispersed by collagenase to prepare lung digests (LD) (Beamer et al., 2010; Thakur et al., 2009). Cells were enumerated using a Coulter Counter (Beckman Coulter, Brea, CA) and prepared for multicolor flow cytometric analysis as described below. 

Flow cytometry. Single cell suspensions from WLL or LD (see above) as specified were washed in complete RPMI and resuspended in 50 µl of purified rat anti-mouse CD16/CD32 diluted 1:100 in PBS with 1% bovine serum albumin and 0.1% sodium azide (PAB) for 15 min on ice to block nonspecific Ab binding. Monoclonal Abs specific to CD11c (APC), clone #HL3), CD11b (Alexa Fluor 700, clone #M1/70), Gr-1-Ly-6C (Alexa Fluor 750, clone #RB6-8C5), F4-80 (PerCp Cy5.3, clone BM-8), and major histocompatibility complex (MHC) class II (PE, clones #NMR-4) were purchased from eBioscience for the analysis of dendritic cells (DCs) and neutrophils. One microgram of each Ab was added to 10^6 total cells and allowed to incubate for 30 min in the dark on ice, with agitation 2–3 times. Finally, cells were washed twice with PBS and resuspended in 0.4 ml PAB. Cell acquisition and analysis were performed on a FACS Aria flow cytometer using FACS Diva software (version 4.1.2, Becton Dickinson). Compensation of the spectral overlap for each fluorochrome was done by gating using anti-rat/hamster Ig compensation beads (BD Biosciences).

Cytokine/chemokine ELISAs. IL-1β, IL-6, IL-10, interferon-γ (IFNγ), monocyte chemotactic protein 1 (MCP-1), and Keratinocyte-derived chemokine (KC) were measured in clarified WLL and tissue culture supernatants as specified in the Results section and figure legends using murine ELISAs kits according to the manufacturer’s instructions and assay procedure (R&D Systems). Color development was assessed at 450 nm on a plate reader.

Pulmonary function assessments. Transpulmonary resistance (Rt) and dynamic compliance (Cdyn) were assessed as previously described (Wells et al., 2008). Mice were challenged with vehicle, followed by increasing concentrations of methacholine (1.5, 3, 6, 12, and 24 mg/ml). Aerosols were generated with an ultrasonic nebulizer (Aeroneb Laboratory Nebulizer; Buxco Electronics, Inc., Troy, NY). A computer program (BioSystemXA; Buxco Electronics) was used to calculate Rt and Cdyn.

Histopathological evaluation of inflammation and fibrosis. Silica-exposed mice were transcardially perfused with PBS and 4% paraformaldehyde-PBS and postfixed overnight at 4°C. Routine histological procedures were used to paraffin embed the lungs. As previously described, 5- to 7-μm sections were cut, mounted on superfast* slides (VWR), and stained with Gomori’s trichrome (EMD Chemicals, Gibbstown, NJ) (Beamer and Holian, 2005; Beamer et al., 2010; Thakur et al., 2009). Five to six mice per group were examined microscopically and representative images captured with a Nikon E-800 microscope and Nuance multispectral camera connected to a Dell computer.

Generation and stimulation of bone marrow–derived macrophages. Bone marrow was aspirated from the femurs and tibiae of C57Bl/6 or AhR−/− mice (6–8 weeks) using a 3-ml syringe filled with RPMI culture media and seeded in 75 cm² tissue culture flasks. Following overnight stromal cell elimination, 20 × 10^6 nonadherent cells were transferred to new flasks, including murine recombinant granulocyte-macrophage colony–stimulating factor (final concentration = 50 ng/ml, R&D Systems Minneapolis, MN). By 7 days, cells were fully differentiated, >75% confluent, and immunopositive for macrophage characteristics as assessed by flow cytometry (data not shown). Viability was determined to be > 90% by trypan blue exclusion staining prior to experimental manipulations. BMM were seeded at 10^6 cells/ml/well of a six-well plate and immediately exposed to vehicle or SiO₂ (100 µg/ml) plus or minus LPS (endotoxin, 1 µg/ml; Sigma-Aldrich), and allowed to incubate for 24 h at 37°C. Following stimulation, BMM were lightly scraped within the spent culture media, centrifuged, and the supernatant and cells separated for analysis. Alternate experiments included pretreatment with 20 ng/ml recombinant murine IL-10 (eBioscience) for 4 h or simultaneous exposure to 20μM caspase-1 inhibitor Ac-Tyr-Val-Ala-Asp-CMK (Cayman Chemical, Ann Arbor, MI). Additional experiments included simultaneous exposure to 0.1% DMSO, 10mM TCDD, SiO₂, or LPS alone or in combination for 24 h using bone marrow macrophages derived from both C57Bl/6 and AhR−/− mice.

RNA isolation and real-time PCR. Bone marrow–derived macrophages were pooled (n = 3, three replicates each), and RNA isolated using the TRizol method as described (Beamer et al., 2010). RNA samples were reverse transcribed using QScript Supermix (Quanta Biosciences, Gaithersburg, MD) and subjected to real-time PCR (RT-PCR) using PerfeCTa SYBR Green Supermix (Quanta Biosciences) in a Stratagene Mx3005p instrument (Agilent Technologies, Santa Clara, CA) (Thakur et al., 2009).

Biochemical quantification of collagen content. Total collagen of the chronic exposure mice was quantified by hydroxyproline as previously described (Beamer et al., 2010). Briefly, lung tissue was excised, weighed, and immediately homogenized using a Tissue-Tearor in sterile water. An aliquot of lung homogenate was hydrolyzed in 12 N HCl at 110°C for 24 h. The mixture was reacted with chloramine T and Ehrlich’s reagent to produce a hydroxyproline-chromophore that was quantified by 550 nm spectrophotometry. Hydroxyproline content for each lobe was determined by triplicate analysis of the sample to provide a mean value.

Statistical analysis. For each parameter, the values for individual mice were averaged and the SD and SE calculated. The significance of the differences between the exposure groups was determined by t-test, one-way, or two-way ANOVA, in conjunction with Tukey’s test for variance, where appropriate. All ANOVA models were performed with Prism software, version 4. A p value of < 0.05 was considered significant.

RESULTS

Increased Susceptibility of AhR−/− Mice to Acute Inflammation

Infiltration of immune cells is an important step in pulmonary inflammation following SiO₂ exposure, and enhanced neutrophilia is a classic marker of SiO₂–induced inflammatory response (Lagasse and Weissman, 1996). To investigate the function of the AhR in particulate-induced acute respiratory inflammation in vivo, we performed studies of experimental silicosis. For these studies, 6- to 8-week-old C57Bl/6 wild-type and AhR−/− mice were exposed to 1 mg SiO₂ or vehicle and inflammation assessed between 1 day and 1 month as described in Supplementary figure 1. At 1, 3, and 7 days, but not 1 month after SiO₂ exposure, AhR−/− mice showed an increase in the total number of lavageable cells compared with C57Bl/6 mice (Fig. 1A). To identify the cell type contributing to the increased cellularity, differential analysis of Wright-Giemsa–stained lavage cells from SiO₂-exposed mice was performed and revealed that AhR−/− mice presented with an exacerbated neutrophilia compared with C57Bl/6 mice (Fig. 1B). To explain the increased sensitivity of AhR−/− mice to respiratory inflammation, the concentrations of several inflammatory cytokines and chemokines in the WLL were assessed 3 days after SiO₂ exposure.
Consistent with the increased susceptibility of AhR<sup>−/−</sup> mice to SiO<sub>2</sub>-induced inflammation, AhR<sup>−/−</sup> mice showed elevated levels of IL-1β, IL-6, MCP-1, and KC, yet decreased levels of IL-10 and IFNγ (Fig. 2). Together, these data indicate that AhR<sup>−/−</sup> mice are hypersensitive to acute inflammation in response to SiO<sub>2</sub> exposure and suggest that activated AhR is contributing toward the regulation of inflammation in response to SiO<sub>2</sub>.

**Chronic Inflammation and Fibrosis in AhR<sup>−/−</sup> Mice**

To assess whether the acute increase in inflammatory cells and mediators correlated with chronic pathology, histopathological

![Image of graphs showing cytokine levels](https://example.com/ahr_regulates_silica-induced_inflammation)

**FIG. 1.** Cellular response to SiO<sub>2</sub> exposure in AhR<sup>−/−</sup> and C57Bl/6 mice. Crystalline silica (SiO<sub>2</sub>) increased the number of cells recovered via WLL in AhR<sup>−/−</sup> compared with C57Bl/6 mice (A). Alveolar inflammation dominated by PMNs was observed—a response that was augmented in SiO<sub>2</sub>-exposed AhR<sup>−/−</sup> mice (B); n = 5–7, values are means ± SEM; *p < 0.05 compared with wild-type mice.

**FIG. 2.** Expression of cytokines and chemokines in SiO<sub>2</sub>-exposed AhR<sup>−/−</sup> and C57Bl/6 mice 3 days postinstillation. Levels of IL-1β, IL-6, MCP-1, and KC were increased, whereas levels of IL-10 and IFNγ were decreased in the WLL fluid after SiO<sub>2</sub> exposure in AhR<sup>−/−</sup> and wild-type C57Bl/6 mice. These responses were exacerbated in AhR<sup>−/−</sup> mice compared with wild-type mice; n = 5–8, values are means ± SEM; *p < 0.05 compared with vehicle control.
assessments of tissue sections from SiO2-exposed lung were performed. For these studies, 6- to 8-week-old C57Bl/6 and AhR−/− mice were exposed to 1 mg SiO2 or vehicle once a week for 4 weeks and inflammation assessed at 1 month as described in Supplementary figure 1. Lung wet weight was higher in SiO2-exposed C57Bl/6 (1.59-fold) and AhR−/− mice (1.42-fold) compared with their respective vehicle-exposed mice indicating the presence of either edema or infiltration of inflammatory cells (Table 1) (Thakur et al., 2009). Representative tissue sections from vehicle-exposed C57Bl/6 and AhR−/− mice showed normal tissue architecture, indicating that the absence of the AhR does not lead to gross anatomical changes in the lungs (data not shown). Histopathology revealed typical focal lesions, interstitial thickening with increased connective tissue matrix, and filling of the air space with cells in SiO2-exposed C57Bl/6 mice (data not shown). In comparison, AhR−/− mice demonstrated an increased accumulation of inflammatory cells (data not shown). Concurrently, fibrosis was assessed by hydroxyproline quantification of the left lung lobe. SiO2-exposed C57Bl/6 (1.75-fold increase) and AhR−/− (1.32-fold increase) mice exhibited increased collagen deposition relative to their respective vehicle-exposed mice, although this response was attenuated in AhR−/− mice (Table 1). Together, these results indicate that AhR−/− mice show increased chronic inflammation but a relative reduction in fibrosis compared with C57Bl/6 mice, emphasizing the critical role of AhR in silicosis.

**AhR Activation in Silicosis**

To evaluate whether AhR activation alters inflammation and fibrosis, C57Bl/6 mice were exposed to SiO2 and the prototypical AhR ligand TCDD either simultaneously or sequentially. In the sequential exposure paradigm, mice were exposed to vehicle or SiO2 once a week for 4 weeks prior to weekly vehicle or TCDD oral gavage for the next 8 weeks as described in Supplementary figure 1. In the concomitant exposure paradigm, mice were gavaged weekly with vehicle or TCDD beginning 1 day prior to vehicle or SiO2 exposure and continuing for 12 weeks as described in Supplementary figure 1. To investigate the association between chronic SiO2-induced inflammation and pulmonary function, airway responsiveness to inhaled methacholine was assessed via transpulmonary resistance ($R_L$) and dynamic compliance ($C_{dyn}$) measures (Glaab et al., 2007). Together, these parameters quantify changes in lung capacity and compliance in disease models (Vanoirbeek et al., 2009). As anticipated, methacholine challenge resulted in dose-dependent increases in $R_L$ and decreases in $C_{dyn}$ with SiO2 exposure exerting significant effects on both measures; however, TCDD treatment did not alter these responses (data not shown) indicating that the activation of AhR-signaling pathways did not modify airway responsiveness to SiO2.

After chronic SiO2 exposure, C57Bl/6 mice exhibited an increase in the total number of lavageable cells as well as the total number of interstitial leukocytes recovered from the LD. These increased cellular infiltrations were reduced by either simultaneous (Figs. 3A and C) or sequential (data not shown) activation of the AhR. To identify the cell type contributing to the increased cellularity of WLL, cell differential analysis of Wright-Giemsa–stained lung sections revealed that chronic SiO2 exposure resulted in an exacerbated neutrophilia as measured by increases in the percent and absolute number of polymorphonuclear cells neutrophils (PMNs)—an event that could be reduced by TCDD (Fig. 3B). To identify the cell type contributing to the increased cellularity in the LD, interstitial leukocytes were stained for cell surface markers to differentiate interstitial DCs (CD11c+CD11b+MHCIIhi) and neutrophils (CD11c–CD11b+Gr1+) (Vermaelen and Pauwels, 2004). Although both the percent and absolute number of interstitial DCs were increased in response to chronic SiO2 exposure, neither parameter was affected by AhR activation (Fig. 3E). TCDD treatment diminished the expression of MHC class II on interstitial DCs relative to vehicle controls (Fig. 3F). In contrast, the absolute number of neutrophils increased in response to chronic SiO2 exposure and was attenuated by activation of the AhR to near baseline levels (Fig. 3D).

To determine if the observed reduction in inflammatory burden following AhR activation correlated with a similar reduction in collagen deposition, fibrosis was assessed by hydroxyproline quantification and Trichrome staining. As anticipated, exposure to SiO2 increased collagen content relative to vehicle control in both sequential and concomitant exposure paradigms. Neither sequential nor concomitant AhR activation by TCDD had a favorable effect on SiO2-induced collagen deposition.

**TABLE 1**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Wet weight</th>
<th>% Increase</th>
<th>Hydroxyproline</th>
<th>% Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Silica</td>
<td>% Increase</td>
<td>Vehicle</td>
</tr>
<tr>
<td>C57Bl/6</td>
<td>92.70 ± 9.7</td>
<td>147.57 ± 6.0*</td>
<td>44.81 ± 5.1</td>
<td>18.98 ± 1.6</td>
</tr>
<tr>
<td>AhR−/−</td>
<td>106.16 ± 2.6</td>
<td>151.19 ± 4.1*</td>
<td>31.54 ± 9.0*</td>
<td>24.62 ± 0.4*</td>
</tr>
</tbody>
</table>

*Note. Chronic SiO2 exposure resulted in increased lung wet weight as well as collagen deposition in the lungs of AhR−/− and C57Bl/6 mice, although this response was attenuated in AhR−/− mice compared with wild type. Values are means ± SEM.

*<i>p < 0.05 compared with wild-type baseline value</i>, *<i>p < 0.05 compared with respective vehicle control</i>, #<i>p < 0.05 compared with percent increase of wild-type mice</i>. n = 5–6.
deposition at 12 weeks and may even have exacerbated fibrosis (Table 2). Similarly, AhR activation had no beneficial effect on SiO2-induced increases in wet weight (Table 2). Representative lung tissue sections from saline + peanut oil–treated mice (Fig. 4A) and saline + TCDD–treated mice (Fig. 4C) showed normal tissue architecture indicating that neither vehicle nor the activation of the AhR leads to gross anatomical changes in the lungs. A typical inflammatory response and thickening of interstitium were observed in peanut oil–treated SiO2–exposed mice (Fig. 4B). In comparison, TCDD-treated mice demonstrated a decreased accumulation of inflammatory cells and similar collagen deposition to peanut oil–treated mice (Fig. 4D). To examine the effect of AhR activation on cytokine production, levels of IL-1β, IL-6, and IL-22 in the WLL were assessed. Consistent with the increased presence of PMNs in the airways and interstitium following chronic SiO2 exposure, elevated levels of IL-1β and IL-6 were observed. However, although AhR activation by TCDD reduced levels of IL-1β in both sequential and concomitant exposure paradigms, AhR activation appeared to augment IL-6 and IL-22 production (Fig. 5). Taken together, these results further emphasize the critical role of the AhR in SiO2-induced inflammation but not fibrosis.

**FIG. 3.** Innate immune responses to SiO2 exposure in TCDD-induced AhR–activated C57Bl/6 mice. Chronic SiO2 exposure increased the total number of lavageable cells (A), and interstitial leukocytes recovered from the lung (C). This increased cellularity was reduced by concomitant activation of the AhR. Chronic SiO2 exposure increased the percent and absolute number of neutrophils (PMNs) in the WLL (B) and LD (D)—events that were inhibited by TCDD-induced AhR activation. Although the percent and absolute number of interstitial DCs were increased in response to chronic SiO2 exposure, neither parameter was affected by AhR activation (E). However, TCDD attenuated MHC class II expression on interstitial DCs (F); n = 5–6, values are means ± SEM; *p < 0.05 compared with vehicle.
TABLE 2
Chronic Inflammation and Fibrosis in TCDD-Treated SiO₂–Exposed Mice

<table>
<thead>
<tr>
<th>Wet weight</th>
<th>Hydroxyproline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle + peanut oil</td>
</tr>
<tr>
<td>Sequential</td>
<td>126.0 ± 3.7</td>
</tr>
<tr>
<td>Concomitant</td>
<td>102.7 ± 5.0</td>
</tr>
</tbody>
</table>

Notes. Neither sequential nor concomitant exposure to TCDD altered SiO₂-induced increases in wet weight and collagen deposition in the lungs of C57Bl/6 mice. In the sequential exposure paradigm, mice were exposed to 25 μl vehicle or 1 mg SiO₂ suspended in vehicle once a week for 4 weeks prior to weekly vehicle (200 μl peanut oil) or TCDD (10 mg/kg) oral gavage for the next 8 weeks. In the concomitant exposure paradigm, mice were gavaged weekly with vehicle or TCDD, beginning 1 day prior to being exposed to vehicle or SiO₂ suspended in vehicle as described in Supplementary figure 1. Values are means ± SEM. *p < 0.05 compared with vehicle controls, n = 5–6.

Enhanced Cytokine Secretion From AhR<sup>−/−</sup> Bone Marrow–Derived Macrophages in Response to Inflammasome Activation

To further investigate the cause of the aberrant cytokine secretion by SiO₂-exposed AhR<sup>−/−</sup> mice, we next assessed if there were any differences in the production of proinflammatory cytokines by wild-type and AhR<sup>−/−</sup> BMM in response to SiO₂. Macrophages derived from the bone marrow of wild-type and AhR<sup>−/−</sup> mice were exposed to SiO₂ and LPS (stimuli that activate the inflammasome), and the levels of IL-1β, IL-6, MCP-1, KC, IL-10, and IFNγ assessed by ELISA. Compared with the levels in wild-type BMM, the levels of IL-1β, IL-6, MCP-1, KC, and IFNγ but not IL-10 secretion by AhR<sup>−/−</sup>BMM were markedly elevated in response to SiO₂-induced inflammasome activation (Fig. 6). Because it is known that macrophages produce IL-10 to regulate the overproduction of inflammatory cytokines (Moore et al., 2001) and autocrine IL-10 feedback diminished inflammasome activation and IL-1β production (Guarda et al., 2011), we tested whether 4 h pretreatment with 20 ng/ml recombinant mouse IL-10 affected the levels of inflammatory cytokines in wild-type and AhR<sup>−/−</sup>BMM following SiO₂ + LPS stimulation. Indeed, secretion of IL-1β, IL-6, MCP-1, and KC but not IFNγ secretion was suppressed by IL-10 (Fig. 6). Together, these results demonstrate that the AhR has an antiinflammatory function in macrophages that may be linked to IL-10 and downstream signaling events such as STAT phosphorylation (Guarda et al., 2011; Kimura et al., 2004).

Expression of AhR-Dependent Genes in Bone Marrow–Derived Macrophages

We next performed RT-PCR analysis of wild-type and AhR<sup>−/−</sup> mouse BMM to evaluate AhR-dependent changes in gene expression that may be related to inflammasome activation and IL-1β secretion. Adapter molecules that could act by disrupting inflammasome functionality (Taxman et al., 2010) include, but are not limited to, inhibitors of PYCARD (Bedoya et al., 2007), the serpin proteinase inhibitor PI-9 (Young et al., 2000), caspase-12 (Saleh et al., 2004, 2006), caspase-1 inhibitors, cathepsin B inhibitors, plasminogen activator inhibitor type 2 (Pai-2), Bcl-2, and Bcl-X<sub>L</sub> (Bruey et al., 2007; Greten et al., 2007). Among the genes whose expression was reduced in AhR<sup>−/−</sup> BMM, we observed markedly reduced levels of Pai-2 and STAT2. Consistent with our ELISA findings both in vitro and in vivo, we observed markedly enhanced levels of IL-1β and IL-6 (Table 3). However, unlike Sekine et al. (2009), we did not find great differences in expression of Bcl-2. The reduced expression of Pai-2 and STAT2 in particular is of interest because they have been reported to negatively regulate IL-1β secretion by inhibiting the activity of caspase-1 (Bruey et al., 2007; Greten et al., 2007). Consistent with the notion that enhanced IL-1β secretion in response to SiO₂ treatment is due to the activation...
of caspase-1, treatment with the caspase-1 inhibitor Ac-Tyr-Val-Ala-Asp-CMK markedly reduced the secretion of IL-1β in AhR−/− BMM (data not shown) as well as the gene expression of pro-IL-1β and STAT2. In contrast, Ac-Tyr-Val-Ala-Asp-CMK increased the relative expression of Pai-2 and Bcl-2 after SiO2 treatment (Table 3). These data suggest that interactions between the AhR and Pai-2 and STAT2 may be one of the causes for the increased inflammatory cytokine production and secretion by AhR−/− BMM after SiO2 treatment.

AhR Activation With TCDD Attenuates Inflammasome-Dependent IL-1β Production In Vitro

Finally, to further investigate the role of the AhR in inflammasome activation, we determined if there were any differences in IL-1β levels present in tissue culture supernatants of wild-type and AhR−/− BMM upon stimulation with vehicle, DMSO, TCDD, SiO2, and LPS alone or in combination. Compared with the levels observed in wild-type BMM, the levels of IL-1β secretion by AhR−/− BMM were markedly elevated—consistent with our results presented in Figure 6. Concomitant treatment with 10nM TCDD in wild-type BMM reduced IL-1β levels relative to the respective vehicle-treated controls; however, these changes were not observed in TCDD-treated BMM derived from AhR−/− mice.

DISCUSSION

A growing number of studies have examined the effects of AhR activation on alterations in specific immune responses, especially with regard to the development of regulatory T cells and Th17 cells (Esser et al., 2009; Kimura et al., 2009; Marshall and Kerkvliet, 2010; Sekine et al., 2009; Stockinger et al., 2011). Close examination of AhR activation as well as studies of AhR−/− mice revealed that they suffer from impaired immunity and more easily succumb to respiratory infections (Jin et al., 2010; Kimura et al., 2009; Sekine et al., 2009; Vorderstrasse et al., 2004). We examined the susceptibility of AhR−/− mice to silicosis and found that they were hyper-sensitive to SiO2-induced inflammation and had increased secretion of inflammatory cytokines and chemokines (Figs. 1 and 2) but not fibrosis (Table 1). Furthermore, macrophages derived from AhR−/− mice secreted enhanced amounts of cytokines and chemokines in response to SiO2 compared with wild-type mice—events which could be reversed by pretreatment with 20 ng/ml recombinant IL-10 (Fig. 6). Analysis of gene expression revealed that AhR−/− macrophages exhibit increased levels of pro-IL-1β, IL-6, and Bcl-2, yet decreased levels of STAT2, STAT5a, and serpin B2 (Pai-2) in response to SiO2 (Table 3). Finally, TCDD induced activation of the AhR in wild-type, but not AhR−/− BMM reduced inflammasome-mediated IL-1β production (Fig. 7). Together, these results suggest that AhR functions as a negative regulator of SiO2-induced inflammation, and an appropriate AhR ligand may be useful for treating patients with inflammatory disorders of the lung.
Despite existing occupational health and safety standards, silicosis remains a prevalent problem throughout the world. Therefore, experimental silicosis remains a valuable tool to study the mechanisms and interactions between cellular and soluble mediators involved in respiratory disease processes. An important first step in acute pulmonary inflammatory response to inhaled SiO₂ particles involves an influx of inflammatory cells (Bowden and Adamson, 1984). As noted previously, C57Bl/6 wild-type mice experience an initial loss in cell number at 1 day postinstillation (Beamer et al., 2010), whereas at later time

![Cytokine response in BMM derived from wild-type and AhR⁻/⁻ mice. Twenty-four hours following SiO₂ + LPS treatment, the levels of IL-1β (A), IL-6 (B), MCP-1 (C), KC (D), and IFNγ were significantly increased in tissue culture supernatants. AhR⁻/⁻ BMM showed enhanced levels of inflammatory cytokines and chemokines compared with wild-type mice. These responses were inhibited by 4 h pretreatment with 20 ng/ml recombinant mouse IL-10; n = 3–5, values are means ± SEM; *p < 0.05 compared with vehicle control. #p < 0.05 compared to SiO₂ + LPS.](image)

**TABLE 3**

<table>
<thead>
<tr>
<th></th>
<th>Pro-IL-1β</th>
<th>IL-6</th>
<th>STAT2</th>
<th>STAT5a</th>
<th>Pai-2</th>
<th>Bcl-2</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>silica + LPS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>silica + caspase-1 inhibitor</td>
<td>2179.83</td>
<td>413.00</td>
<td>31.78</td>
<td>30.91</td>
<td>8.69</td>
<td>2.22</td>
</tr>
<tr>
<td>AhR⁻/⁻</td>
<td>3258.52</td>
<td>1606.83</td>
<td>138.14</td>
<td>248.99</td>
<td>4.53</td>
<td>1.20</td>
</tr>
</tbody>
</table>

Note. Bone marrow macrophages derived from AhR⁻/⁻ mice show elevated mRNA levels of pro-IL-1β, IL-6, and Bcl-2, yet decreased mRNA levels of STAT2, STAT5a, and serpin B2 (Pai-2) compared with wild-type C57Bl/6 macrophages in response to Nlrp3 inflammasome activation. Inhibition of caspase-1 activity with 20μM Ac-Tyr-Val-Ala-Asp-CMK showed caspase-1-dependent induction of pro-IL-1β, STAT2, and Bcl-2 in response to Nlrp3 inflammasome activation. Values are expressed as fold change of ΔACT over respective untreated vehicle control, n = 3, pooled.
points, SiO₂ exposure resulted in increased numbers of immune cells—a process which was exaggerated in AhR−/− mice. This initial decrease in cell number may occur as a result of acute migration, cell death, or both. Although this phenomenon has also been observed with other particulates such as wood smoke (unpublished results), further studies are necessary to determine the mechanisms responsible for this cell loss and the role of the AhR in these processes. It should also be noted that recent publications have demonstrated defects in mucosal immune cells including lung γδ T cells and Langerhans cells in conventional AhR knockout mice, and γδ T cells have been implicated in inflammation following silica; thus, the responses to SiO₂ that we observed may be influenced by an inherited altered immune environment in the lung (Jux et al., 2009; Lo Re et al., 2010; Simonian et al., 2010). Because the pulmonary response to inhaled SiO₂ involves increases in antigen-presenting cells, such as macrophages and DCs, and an influx of neutrophils (8, 38), it was critical to determine the cell type responsible for immune cell influx observed in SiO₂-exposed AhR−/− mice. AhR−/− mice show an increased presence of neutrophils within the airways. Neutrophils may play a detrimental role in silicosis by secreting inflammatory enzymes, such as myeloperoxidase, matrix metalloproteinases, and elastase (Hoshino et al., 2000). Inhibition of these functions may therefore represent a promising therapeutic strategy for the treatment of airway inflammatory diseases characterized by exacerbated PMN infiltration and activation. These data show for the first time that AhR−/− mice are hypersensitive to inhaled SiO₂.

Numerous cytokines and chemokines participate in the development and progression of inflammation and fibrosis in the lungs (Castranova et al., 2002; Hamilton et al., 2008; Rao et al., 2004; Rimal et al., 2005; Sumida et al., 2008). Of these, IFNγ, IL-1β, IL-6, IL-10, IL-17, and IL-22 have been shown to be important in the pathogenesis of silicosis (Barbarin et al., 2005; Cassel et al., 2008; Driscoll et al., 1990, 1996; Lo Re et al., 2010; Srivastava et al., 2002). Consistent with the exaggerated influx of PMNs into the alveolar spaces following SiO₂ exposure, increased levels of inflammatory cytokines (IL-1β, IL-6) and chemokines (MCP-1 and KC) but decreased levels of the anti-inflammatory cytokine IL-10 were observed in SiO₂-exposed AhR−/− mice. These data show that the AhR may regulate the acute inflammatory response to inhaled SiO₂. Furthermore, these results demonstrate the dynamic interplay among pro and anti-inflammatory cytokines and also highlight the uncertainty that exists regarding the respective roles of Th1- and Th2-associated cytokines in silicosis. Although comparative analysis of the cytokine environment in the WLL was only performed at 3 days post-SiO₂ instillation, future studies, to provide a more comprehensive assessment of the timing of cytokine expression, may yield important information about the contribution of cytokines to silicosis and the role of the AhR in these events. Our data further imply that attempts to manipulate single cytokines therapeutically may be overly simplistic and ultimately ineffective.

Whereas these results demonstrate that the AhR is involved in regulation of inflammation, little data exist regarding the role of the AhR in development of lung fibrosis in vivo. Duration and amount of silica exposure as well as the content of free SiO₂ are fundamental determinants of silicosis (Lynch and McCune, 1997). However, significant differences in the susceptibility of various inbred mouse strains exist with regard to collagen deposition (Ohtsuka et al., 2006). The current study utilized the most susceptible mouse strain, C57BL/6, for analysis following exposure to SiO₂ because of its relative sensitivity to developing silicosis, its high-affinity AhR, and because it is the appropriate background control strain for the AhR−/− mice. As expected, the combination of hydroxyproline analysis and trichrome staining of lung sections showed significant interstitial inflammation, collagen deposition, and development of fibrosis following SiO₂ exposure in C57BL/6 mice. In contrast, AhR−/− mice exposed to SiO₂ revealed reduced collagen deposition relative to wild-type mice. These findings are particularly interesting in light of the concomitant presence of abnormal numbers of inflammatory cells within the lung tissue of SiO₂-exposed AhR−/− mice—indicating differences in the complex processes of inflammation and fibrosis. Although wild-type mice showed areas of focal inflammation following SiO₂ exposure, these areas were quite small compared with the extensive infiltration of inflammatory cells observed in AhR−/− mice and suggest a disconnect between the complex processes of inflammation and fibrosis similar to our previous findings in other receptor-deficient mouse strains, including MARCO−/−, SRA−/−, Rag1−/−, Rag1−/− NK depleted, IL-1R−/−, and IL-18R−/− (unpublished results) (Beamer and Holiain, 2005; Beamer et al., 2010; Thakur et al., 2009). The failure of AhR−/−
mice to progress from an inflammatory phase to a fibrotic phase conspicuously separates the two events. One possible explanation is the failure to transition from a “Th1” to a “Th2” type of response, as has been proposed by a number of fibrosis models (Izbiicki et al., 2002; Kaviratne et al., 2004; Kolodsiick et al., 2004). Future studies to measure SiO2-induced fibrosis in AhR<sup>−/−</sup> mice at various time points will elucidate if the rate of development of silicosis differs and whether the AhR promotes Th1/Th2 orientation of immune responses over time.

To further investigate the role of the AhR in vivo and to better define its biological activity during SiO2-induced lung inflammation and fibrosis, we analyzed the pulmonary responses to chronic SiO2 exposure in TCDD-treated mice. Analysis of WLL and LD cells showed chronic SiO2 exposure results in an exacerbated neutrophil-dominated response, which was reduced by exposure to TCDD. These responses are in contrast to findings by Teske et al. (2005) where exposure to TCDD resulted in a surfeit of neutrophils in mice infected with influenza A virus. Although AhR activation clearly modulates host responses to respiratory pathogens in diverse ways, we still have much to understand about the complex interactions between immune cells, inhaled particulates, and the host environment.

Respiratory DCs capture antigen, migrate to lymph nodes, and play a key role in activating naive T cells in response to pathogens. Both naive splenic DCs and inflammatory bone marrow–derived DCs display altered surface molecule expression following TCDD-induced AhR activation (Bankoti et al., 2010a,b; Vorderstrasse and Kerkvliet, 2001). Previous reports further demonstrated that AhR activation impairs lung DC migration and reduces the ability of DCs isolated from the mediastinal lymph nodes to activate naive T cells (Jin et al., 2010). Similar to these studies, our data show that AhR stimulation modulates the activation of respiratory DCs in response to SiO2 exposure. Although we observed no effect on either the percent or absolute number of DCs, TCDD-induced AhR activation diminished DC activation in response to SiO2 exposure as measured by MHC class II expression. Because data from bone marrow–derived macrophages demonstrated a significant increase in APC activity following SiO2 exposure in an in vitro antigen presentation assay (Migliaccio et al., 2005), future studies will examine whether TCDD-induced AhR activation may diminish SiO2-induced APC activity of DCs in vitro. Nonetheless, defects in DC function following AhR activation in silicosis could affect particle clearance and potentially alter APC activity and subsequent T-cell–mediated immune responses.

Our results support the notion that the AhR is critical for controlling acute and chronic pulmonary inflammation in response to SiO2. In the lungs, SiO2-induced inflammation is orchestrated by cytokines that recruit inflammatory cells and propagate the inflammatory response as well as promote lung injury (Hamilton et al., 2008; Rao et al., 2004). In particular, IL-1β and IL-6 are critical contributors to silicosis (Cassel et al., 2008; Driscoll et al., 1990; Franchi et al., 2009a,b; Srivastava et al., 2002; Tripathi et al., 2010). In the present study, levels of both cytokines were increased in response to chronic SiO2 exposure, yet only IL-1β levels were diminished in response to TCDD-induced AhR activation. In contrast, SiO2-induced levels of IL-6 and IL-22 were augmented by AhR activation. These results suggest that the AhR modifies cytokine gene expression via divergent signaling pathways such as NF-kB and Jak-STAT (Kimura et al., 2008; Wu et al., 2011). Notably, TCDD affects many physiologic responses beyond cytokine production, and it remains to be determined what potential indirect effects this treatment may have had in the lungs of SiO2-treated mice. Components of IL-22 signaling appear to be involved in lung inflammation and fibrosis, and AhR ligands such as environmental toxins and endogenous breakdown products of aromatic amino acids promote IL-22 expression during immune responses both in vitro and in vivo (Veldhoen and Duarte, 2010; Veldhoen et al., 2008, 2009).

Although a recent report indicated IL-22<sup>+</sup> lymphocytes accumulated in the lungs of SiO2-exposed mice (Lo Ré et al., 2010), the exact contributions of IL-22 to SiO2-induced inflammation and fibrosis remain unclear. Together, these results suggest that although different AhR ligands may contribute to disparity in inflammatory responses to respiratory pathogens, the AhR-signaling pathway may represent a promising novel therapeutic approach to treat patients with inflammatory disorders of the lung.

Because AhR<sup>−/−</sup> mice exhibit enhanced neutrophilia and IL-1β levels and TCDD-induced AhR activation reduced neutrophilia and IL-1β levels in response to SiO2 exposure, we sought to further define the role of the AhR in the regulation of inflammatory responses and inflammasome activation in particular. Consistent with our observations in vivo, in response to SiO2-induced inflammasome activation in vitro, AhR<sup>−/−</sup>BMM secreted much larger amounts of inflammatory cytokines such as IL-1β and chemokines. These responses were inhibited by pretreatment with recombinant mouse IL-10 similar to previous reports (Guarda et al., 2011). In addition, IL-1β messenger RNA (mRNA) levels were markedly elevated in AhR<sup>−/−</sup>BMM compared with wild type, suggesting that the increased IL-1β secretion is likely due to enhanced synthesis and processing of IL-1β. We believe that the exacerbated IL-1β secretion by AhR<sup>−/−</sup>BMM may provide clues as to how AhR functions as a physiological immune suppressor. Real-time PCR analyses to investigate the AhR-dependent changes in gene expression revealed that the levels of expression of IL-1β and IL-6 were markedly increased, whereas STAT2 and Pai-2 mRNA were markedly reduced in AhR<sup>−/−</sup>BMM. These findings are consistent with previously published reports (Kimura et al., 2009; Sekine et al., 2009). There are several pathways for processing IL-1β that lead to its secretion (Martinon and Tschopp, 2004). SiO2 induced IL-1β processing that is regulated by the Nlrp3 inflammasome involves caspase-1 (Cassel et al., 2008; Martinon and Tschopp, 2007). Consistent
with these observations, treatment with the caspase-1 inhibitors, Ac-Tyr-Val-Ala-Asp-CMK not only reduced the secretion of IL-1β in AhR−/− BMM but also reduced IL-1β gene expression. Finally, our results demonstrated that TCDD-induced activation of the AhR in wild type but not AhR−/− BMM reduced inflammasome-mediated IL-1β production (Fig. 7). Further investigation is necessary to establish the molecular basis of AhR inflammasome regulation of IL-1β secretion.

In summary, this study highlights the importance of the AhR in crystalline silica–induced pathology. First, AhR−/− mice exhibited increases in acute and chronic inflammation following SiO₂ exposure yet only slightly increased fibrosis. Second, AhR activation reduced SiO₂-induced lung injury but slightly increased fibrosis. Together, these findings provide evidence of an important role of the AhR in vivo in regulation of SiO₂-induced inflammatory responses. Third, in vitro assays confirm the heightened inflammatory capacity of AhR-deficient macrophages to respond to crystalline SiO₂ and that this response could be abrogated by TCDD-induced activation of the AhR. Finally, altered gene expression profiles in AhR−/− BMM suggest possible signal transduction mechanisms of whereby AhR ligands might regulate inflammation.

The recent identification of the AhR as a master regulator of mucosal barrier function and the significance of AhR-mediated responses following activation by a variety of endogenous and exogenous ligands suggest that alterations in AhR expression or function may influence the inflammatory response in lung disease (Stejskalova et al., 2011). Currently, a significant area of AhR biology is directed toward the identification of existing and/or the generation of novel compounds that can bind and activate the AhR for beneficial therapeutic outcomes. For example, natural/endogenous AhR agonists, such as 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester, VAG539, indole-3-carbinol, and 3,3'-diindolylmethane, have recently been shown to have beneficial effects in several immune-mediated diseases including experimental autoimmune encephalitis (a murine model of multiple sclerosis), allograft tolerance, 2,4,6-trinitrobenzene sulfonic acid-induced colitis (unpublished data), and experimental arthritis, respectively (Dong et al., 2010; Hauben et al., 2008; Quintana et al., 2010). Several of these effects have been directly linked to effects in specific immune cell populations such as DCs and regulatory T cells, although therapeutic effects following AhR activation in vivo may ultimately prove to be due to collective effects in many immune and nonimmune cell populations. It is not the intent of our current study to suggest that TCDD be used clinically to treat humans. However, it is expected that TCDD-like chemicals with high affinity for the AhR but limited associated toxicity (as observed with natural/endogenous AhR agonists) will have significant therapeutic effects when used to treat autoimmune and chronic inflammatory diseases, including immune-mediated lung diseases.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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AhR regulates silica-induced inflammation


