Scavenger receptor class A type I/II (CD204) null mice fail to develop fibrosis following silica exposure

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Beamer, Celine A., and Andrij Holian. Scavenger receptor class A type I/II (CD204) null mice fail to develop fibrosis following silica exposure. Am J Physiol Lung Cell Mol Physiol 289: L186–L195, 2005. First published April 22, 2005; doi:10.1152/ajplung.00474.2004.—Alveolar macrophages express the class A scavenger receptor (CD204) (Babaev VR, Gleaves LA, Carter KJ, Suzuki H, Kodama T, Fazio S, and Linton MF. Arterioscler Thromb Vasc Biol 20: 2593–2599, 2000); yet its role in vivo in lung defense against environmental particles has not been clearly defined. In the current study, CD204 null mice (129Sv background) were used to investigate the link between CD204 and downstream events of inflammation and fibrosis following silica exposure in vivo. CD204+/− macrophages were shown to recognize and uptake silica in vitro, although this response was attenuated compared with 129Sv wild-type mice. The production of tumor necrosis factor-α in lavage fluid was significantly enhanced in CD204 null mice compared with wild-type mice following silica exposure. Moreover, after exposure to environmental particles, CD204+/− macrophages exhibited improved cell viability in a dose-dependent manner compared with wild-type macrophages. Finally, histopathology from a murine model of chronic silicosis in 129Sv wild-type mice displayed typical focal lesions, interstitial thickening with increased connective tissue matrix, and cellular infiltrate into air space. In contrast, CD204+/− mice exhibited little to no deposition of collagen, yet they demonstrated enhanced accumulation of inflammatory cells largely composed of neutrophils. Our findings point to an important role of CD204 in mounting an efficient and appropriately regulated immune response against inhaled particles. Furthermore, these results indicate that the functions of CD204 are critical to the development of fibrosis and the resolution of inflammation.

inflammation; alveolar macrophages

THE RESPIRATORY TRACT is constantly exposed to potentially pathogenic particles and microorganisms. In the lung, the alveolar macrophage (AM) is primarily responsible for the binding, ingestion, and clearance of inhaled particulate matter (15). The AM response to inhaled particulates ranges from ingestion and clearance with minimal inflammation to profuse production of inflammatory cytokines and reactive oxygen intermediates (1, 10, 61). The former capacity is particularly useful in the clearance of particulates without risk of the potentially damaging effects of an unnecessary inflammatory response. Consequently, the array of receptors expressed on the surface of AM is critical for recognition, binding, uptake, and response to inhaled environmental particulates in the maintenance of lung homeostasis.

One family of receptors important for normal host defense within the lungs is the scavenger receptors (SR), which includes classes A through F, and an “other” class that includes such receptors as RAGE, FEEL-1, and FEEL-2 (23). Macrophage scavenger receptors are trimeric integral membrane glycoproteins whose extracellular domains include α-helical coiled-coil, collagenous, and globular structures (33, 52). SR recognize a diverse array of ligands that may be loosely grouped into two categories: polyanionic compounds (e.g., polyinosinic acid, fucoidan, lipotechoic acid) and modified proteins [e.g., acetylated low-density lipoprotein (AcLDL), maleylated albumin, glycated collagen] (26). Other naturally occurring ligands include polynucleotides and polysaccharides, gram-positive and gram-negative bacteria, and apoptotic cells (16, 53, 67). The versatile biological functions of SR include the clearance of pathological substances, host defense, cell adhesion, activation of intracellular signaling events, and cytokine production (21, 26, 50, 51, 57, 65), although it is not yet clear how SR coordinate these immune and homeostatic functions.

The class A macrophage SR include four members: types I, II, and III and macrophage receptor with collagenous structure (MARCO) (23) and generally exhibit the capacity to bind and endocytose large quantities of modified lipoprotein (e.g., AcLDL and oxidized LDL) (23, 43). The first identified member of the class A SR, CD204, is a homotrimeric glycoprotein composed of three 77-kDa monomers generated from alternative splicing of a message encoded by a single gene (33, 52). CD204 is present on tissue macrophages of the spleen, thymus, liver, heart, and gut (48), and CD204 activity is found in liver and lung (19, 25, 26). In vitro, CD204 expression by murine peritoneal macrophages is upregulated by macrophage colony-stimulating factor (M-CSF) and downregulated by LPS, TNF-α, or IFN-γ (18, 44, 64), although the regulation of CD204 on macrophage populations in vivo remains poorly understood. Class A SR are believed to play a role in multiple pathophysiological processes, including atherosclerosis and host defense through ligand internalization and promotion of cell adhesion (43). Furthermore, studies have begun to explore the role of CD204 activation and its intracellular signaling ability to modulate these diverse functions in macrophages (11, 24, 32, 41, 43).

SR may mediate the physiological and pathological interactions of inhaled particulate matter with AM. Previous work has identified a specific role for CD204 in the regulation of silica-induced apoptosis (20) and further elucidated that SR are...
necessary for caspase activation and subsequent apoptosis (as well as necrosis) caused by silica in a macrophage-like cell line (9). Additional work has demonstrated the ability of human AM to undergo apoptosis in response to silica exposure in vitro and that blocking with a CD204 ligand-binding inhibitor ablated this response (27).

The current study was undertaken to more precisely evaluate the role of CD204 in the development of inflammation and fibrosis. In vitro studies examined the role of CD204 in recognition and phagocytosis of silica by comparing macrophages from CD204 null and 129Sv wild-type mice. Cell fate following particulate exposure was analyzed using bone marrow-derived macrophages from 129Sv wild-type and CD204−/− mice. Additional experiments examined the pulmonary response to crystalline silica using histomorphometric means in a murine model of chronic silicosis.

**MATERIALS AND METHODS**

**Mice.** Initial breeding pairs of CD204 null mice were generously provided by Dr. Willem J. S. de Villiers (Univ. of Kentucky Medical Center). In addition, the founding 129Sv wild-type mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice used in these studies were propagated by homozygous mating colonies and maintained in microisolator units in the University of Montana specific pathogen-free animal facility. Cages, bedding, and food were sterilized by autoclaving, and mice were handled with aseptic forceps or aseptic gloves. Mice were allowed food and water ad libitum and were used experimentally at 6–8 wk of age. All animal use procedures were in accordance with National Institutes of Health and approved by the University of Montana institutional animal care and use committee.

Identification of CD204 null mice. DNA samples were extracted from mouse tail and amplified using forward (5’-CAAGTGATACATCTCAAAGTCT-3’), reverse (5’-CTGTAGATCCAGGACTCTG-3’), and Neo insert (5’-GAGGAGTAGAAGGTGGCCGGCAA-3’) primers (Operon Technologies, Alameda, CA) encompassing the site of the SR A I/II and Neo insert (5). Amplification was performed in a PTC-200 Gradient Cycler (MJ Research, Las Vegas, NV) with the following parameters: 1 min 94°C, 1 min 60°C, 1 min 72°C and subjected to 35 cycles of PCR. Genotyping was determined by separation of the amplified PCR products by 1.5% agarose gel electrophoresis at 75 V for 2.5 h. 129Sv wild-type mice show a 440-bp band, whereas CD204 null mice show a 325-bp band.

Harvest of AM and quantification of silica uptake. Unexposed 129Sv wild-type and CD204−/− mice (n = 5) were anesthetized with a lethal dose of pentobarbital sodium, and AM were harvested from the lungs using three 1-mL lavages of PBS with 2% BSA (pH 7.4). Briefly, pooled AM were pelleted at 1,500 rpm for 5 min, and red blood cells were lysed (mouse erythrocyte lysis solution, R&D Systems), washed twice in PBS, and resuspended in 1 ml of warm RPMI media supplemented with 10% FCS, antibiotic/antimycotic solution, 2-mercaptoethanol, and sodium pyruvate. The trypan blue exclusion method was used to count total number of viable cells. AM were then resuspended in fresh media containing 100 μg of crystalline silica per 1 × 10^6 AM per 1 ml of media and placed in an incubator at 37°C for 4 h. After silica exposure, non-specific antibody binding of AM was blocked by the addition of 30 μg of rat IgG to each sample before staining with optimal concentrations of fluorochrome-conjugated antibody. Two micrograms of F4-80-PE (Serotec) and CD11c FITC (BDPharmingen, San Jose, CA) were added to the cells, and they were allowed to incubate for 30 min at room temperature, with agitation two to three times. Finally, AM were washed twice with PBS, resuspended in 0.4 ml of PBS+azide+BSA, and analyzed immediately on a fluorescence-activated cell sorting (FACS) Aria flow cytometer using FACS Diva software (Becton Dickinson). Each experiment was performed in triplicate. Flow cytometric methods detected silica uptake into the AM by assaying for changes in forward and side scatter properties while gating on AM, which were dual positive for the markers CD11c and F4-80 (13).

**Alveolar lavage and TNF-α analysis.** Wild-type 129Sv and CD204−/− mice (n = 3–4) at 6–8 wk of age were anesthetized with tribromoethanol (0.1 mg/kg) and received a single intranasal instillation of 1 mg of crystalline silica suspended in 30 μl of saline. At 4 and 24 h postinstillation, silica-exposed mice were anesthetized with a lethal dose of pentobarbital sodium. A single 1-ml lavage of PBS (pH 7.4) was utilized to collect lavage fluid and cells from individual mice. Pellet centrifugation permitted collection of lavage fluid (cytokine ELISA) and lavage cells (differential staining and DiffQuik analysis). Unexposed 129Sv wild-type and CD204−/− mice (n = 3–4) were anesthetized, and samples were harvested as indicated above for cytokine ELISA and differential analysis. TNF-α production was assayed using murine TNF-α ELISA kits according to the manufacturer’s instructions and assay procedure (R&D Systems). Color development was assessed at 450 nm on a plate reader.

**Preparation of bone marrow-derived macrophages.** Unexposed 129Sv wild-type and CD204−/− mice at 6–8 wk of age (n = 3) were killed using cervical dislocation, and the femurs and tibias were collected in ice-cold PBS (pH 7.4). In a sterile hood, bone marrow cells were aspirated using a 1-ml syringe filled with RPMI culture media. Aspirated material was centrifuged and resuspended in fresh medium, counted, and seeded to T-75 tissue culture flasks. After stromal cell elimination by overnight adherence, nonadherent cells were transferred to new flasks, and M-CSF (R&D Systems) was added at a final concentration of 20 ng/ml. After 3–4 days, the media were boosted with additional M-CSF (45). By 7 days, the cells were fully differentiated and stained positive for F4-80 and CD11b or CD18 using flow cytometry (macrophage marker). Viability was determined to be >90% by trypan blue exclusion staining.

**Cell titer blue assay.** Bone marrow-derived macrophages from 129Sv wild-type and CD204−/− mice were harvested from T-75 tissue culture flasks using trypsin-EDTA and plated in sterile 96-well microplates at a density of 0.1 × 10^6 cells/well. Cells were allowed to adhere to the plate for 1 h before being exposed to varying concentrations of silica or titanium dioxide particles for 4 h. Silica was obtained from Pennsylvania Glass Sand (Pittsburgh, PA). TiO2 was obtained from Fisher Scientific (Denver, CO). The TiO2 doses used in this study differ from the silica doses to correct for surface area and the number of particles used (e.g., at doses equivalent to silica). The CellTitre-Blue Cell Viability Assay provides a homogeneous, fluorescent method for monitoring cell viability (Promega, Madison, WI). The assay is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin). Nonviable cells rapidly lose metabolic capacity and thus do not generate a fluorescent signal. The homogeneous assay procedure involves adding the single reagent directly to cells cultured in serum-supplemented medium during particle exposure. After an incubation step, data were recorded using a plate-reading fluorometer.

**Silica instillation.** Silica (Min-U-Sil-5, with average particle size 1.5–2 μm) obtained from Pennsylvania Glass Sand was acid washed, dried, and determined to be free of endotoxin by Limulus assay (data not shown; Cambrex, Walkersville, MD). Particulates were suspended in sterile saline and sonicated for 5 min before instillation. CD204−/− (n = 5–8) and wild-type 129Sv (n = 5–8) mice at 6–8 wk of age were anesthetized with tribromoethanol (0.1 mg/kg) and instilled intranasally with 1 mg of crystalline silica suspended in 30 μl of saline once a week for 4 wk. At 6 mo postinstillation, mice were deeply anesthetized, and the lungs were inflated with 1–2 ml of Histochoice (Amresco, Solon, OH). The left lobe of each of the lungs was postfixed for 48 h at 4°C, processed, and embedded in paraffin blocks as previously described (62, 63). Lungs were then serially sectioned at 5 μm and mounted in a 1 in 10 series on Superfrost Plus slides (VWR, West Chester, PA) for further histological analysis.
Histology. For light microscopy, slides were immunostained for CD204 (1:2,000; 2F8 Serotec, Oxford, UK), neutrophils (1:500, NIMP-R14 Hbt), macrophages (1:50, F4-80 Serotec), apoptotic cells (1:500, anti-active caspase-3, Promega), and antibodies, all of which required proteolytic antigen retrieval using 0.02% trypsin (Sigma, St. Louis, MO) with 0.02% Ca^{2+} chloride in distilled water applied directly to the tissue sections, except NIMP-R14 (neutrophils). The standard procedure using the Vector ABC kit was used with appropriate biotinylated secondary antibody diluted 1:500 (Vector Laboratories, Burlingame, CA) and visualized using 3,3′-diaminobenzidine (Sigma) in Tris buffer with 0.001 M imidazole and 0.1% hydrogen peroxide as the chromogen. Slides were then rinsed in water, dehydrated, and coverslipped using DPX mounting media (BDH, Poole, UK). To rule out nonspecific binding of the secondary antisera, control experiments were performed by leaving out the primary antibody (data not shown).

Adjacent sections were stained with Gomori’s trichrome using a Thermo Shandon automated stainer (Shandon, Pittsburgh, PA) to visualize collagen content, as well as the cellular architecture of the lung. Additional sections were stained with the fluorescent dye Lucifer yellow (0.1 mg/ml; Molecular Probes, Eugene, OR) for visualization and quantification of collagen formation. Laser scanning cytometry (LSC) represents an innovative means to rapidly quantify collagen deposition and the development of fibrosis within intact lung sections (3, 4). The fluorescent dye Lucifer yellow appears to selectively stain connective tissue matrix macromolecules, permitting visualization as well as quantification of collagen matrix material using advanced microscopy methods in a manner that yields comparable fibrosis values to the traditional hydroxyproline assay (4).

Image analysis. An LSC (CompuCyte, Cambridge, MA) was used in conjunction with the collagen-specific dye Lucifer yellow to quantify the extent of lung fibrosis based on collagen deposition in intact tissue sections. Quantification of Lucifer yellow staining, using histopathology means, has been previously shown to correlate with the more common hydroxyproline biochemical assay (3, 4, 58). With the use of WinCyte software, the mean pixel intensity per square micrometer was determined based on the assessment of nine random areas within nonsequential tissue sections for each mouse.

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

RESULTS

Silica uptake is attenuated in CD204 null macrophages. To investigate whether the absence of CD204 results in impaired recognition and uptake of silica, 129Sv wild-type and CD204-/- AM were exposed to silica in vitro for 4 h. Changes in side scatter properties that indicate alterations in cellular size and granularity were measured by flow cytometry techniques and used to demonstrate uptake of silica into AM. After silica exposure, AM from 129Sv wild-type mice demonstrated a 2.12-fold significant increase in granularity compared with unexposed AM (Fig. 1). Interestingly, CD204-/- AM were also found to recognize and uptake silica, resulting in a 1.71-fold significant increase in granularity (Fig. 1). Although this increase in granularity observed in CD204-/- AM is significantly attenuated compared with wild-type AM, the data demonstrate that CD204-/- AM recognize and uptake silica during a 4-h period in vitro. These data suggest that CD204 is responsible for a portion of the silica recognition and uptake into a phagocytic cell, and other as yet unknown receptors may be involved.

CD204 modulates macrophage survival in response to silica. Previous work from our laboratory using macrophage cell lines and transfected cells suggested a role for CD204 in the regulation of silica-induced apoptosis (9, 20, 27). To assess whether CD204 mediates cell survival following interactions with inhaled environmental particles, macrophages derived from wild-type and CD204-/- mice were exposed to silica or titanium dioxide at varying doses (µg particle/100,000 cells/well) for 4 h in 96-well culture plates. Cell titer blue fluorometric assays revealed increased cell injury and decreased survival in response to crystalline silica in 129Sv macrophages (Fig. 2). Although CD204-/- macrophages also showed cell injury and decreased survival in response to silica exposure, this response was attenuated compared with 129Sv wild-type macrophages (Fig. 2). Statistical analysis demonstrates that the main effects of strain, particle, and dose were all significantly different. Despite the dose-response curves for silica-exposed macrophages being significantly decreased in 129Sv macrophages compared with CD204-/- macrophages, only the 50-µg dose showed significant effects on CD204-/- macrophage susceptibility to silica-induced cell injury (Fig. 2). Another particle, titanium dioxide, exerted no effect on cell viability in either wild-type or CD204-/- macrophages at any of the doses examined (Fig. 2). These data indicate that cell viability is diminished following silica, but not titanium dioxide exposure and that CD204 is involved in this process.

CD204 null mice develop excessive inflammation following silica exposure. The lungs of untreated CD204-/- and appropriate age-matched 129Sv wild-type mice were examined using histological stains to establish that lack of CD204 does not affect gross cellular architecture within the respiratory system. Close examination of lungs from 129Sv and CD204-/- unexposed mice using Gomori’s trichrome indicated no gross anatomical differences between 129Sv wild-type and CD204-/- lung (Fig. 3, A and B, respectively). Having established baseline
morphology in 129Sv wild-type and CD204−/− mice was utilized in both strains of mice to characterize the pulmonary response at 6 mo postexposure. Histopathology revealed typical focal lesions, interstitial thickening with increased connective tissue matrix, and filling of the air space with cells in the 129Sv wild-type mice (Fig. 3C). In comparison, CD204−/− mice exhibited little to no change in the connective tissue matrix yet demonstrated increased accumulation of inflammatory cells (Fig. 3D). The observation that CD204−/− mice do not appear to develop fibrosis yet show enhanced inflammation compared with 129Sv wild-type suggests that CD204 plays a role in regulating both inflammation and fibrosis.

129Sv wild-type mice develop fibrosis following silica exposure, whereas CD204 null mice do not. Fluorescence microscopy in conjunction with LSC was used to further study the deposition of connective tissue matrix and quantify the development of fibrosis within the lungs of 129Sv wild-type and CD204−/− mice in response to chronic silica exposure. Microscopic examination of lung sections from untreated 129Sv and CD204−/− mice showed similar patterns and intensity of Lucifer yellow staining (Fig. 4, A and B). Lung sections from silica-exposed 129Sv mice appeared to reveal increased intensity of staining for Lucifer yellow in silica-exposed 129Sv mice compared with untreated 129Sv mice (Fig. 4C). In contrast, lung sections from silica-exposed CD204−/− mice were virtually identical to untreated CD204−/− mice (Fig. 4D). Quantitative analysis of the intensity of Lucifer yellow staining using LSC techniques confirmed that 129Sv silica-exposed mice exhibit a significant increase in collagen deposition, indicative of the development of fibrosis, whereas CD204−/− mice show no increase in collagen formation (Fig. 4E). These

Fig. 3. CD204 deficiency does not affect gross cellular architecture within the respiratory system, although CD204 appears to modulate inflammation and fibrosis following silica exposure. A and B: Gomori’s trichrome staining indicates no gross anatomical differences between 129Sv wild-type and CD204−/− lung. C: typical focal lesions, interstitial thickening with increased connective tissue matrix, and filling of the air space with inflammatory cells are observed in silica-exposed 129Sv wild-type mice. D: CD204−/− silica-exposed mice exhibited little to no change in the connective tissue matrix yet demonstrated increased accumulation of inflammatory cells. Scale bar = 50 μm.
findings suggest that CD204 may be involved in the development of fibrosis through an as yet unknown mechanism.

Silica exposure results in expanded neutrophilia in CD204 knockout mice. Information from Gomori’s trichrome staining demonstrated an accumulation of inflammatory cells in the lung tissue of CD204−/− silica-exposed mice, which was absent from 129Sv silica-exposed mice. To determine the cell type responsible for this inflammation, immunostaining for the markers F4-80 (macrophages) and NIMP-R14 (neutrophils) was performed on both untreated and silica-exposed lung tissue. Small focal areas of NIMP-R14 neutrophils and F4-80 macrophages were observed in 129Sv silica-exposed mice, characteristics of silicosis (Fig. 5, A and C, respectively), whereas the vast majority of inflammatory cells within the lung sections of CD204−/− silica-exposed mice were NIMP-R14 neutrophils and not F4-80 macrophages (Fig. 5, B and D, respectively).

During the initial phase of silicosis, the presence of inflammation and fibrosis in both 129Sv wild-type and CD204−/− mice was further evaluated at 1 and 4 wk postexposure. Histological examination revealed similar accumulations of inflammatory cells between the two strains of mice at both time points yet showed no appreciable increase in collagen deposition in either strain of mice (data not shown). These data indicate that the buildup of inflammatory cells within silica-exposed CD204−/− mice occurs over periods of time longer than 4 wk.

A natural progression of inflammation within the alveolar spaces is regulation of polymorphonuclear leukocyte infiltration through controlled apoptosis. To determine the role of CD204 in clearance of apoptotic cells, silica-exposed lungs were immunostained for the markers active caspase-3 and poly(ADP-ribose) polymerase (data not shown) to assay for the presence of apoptotic cells. Although very few cells immunostained positive for apoptosis in either 129Sv wild-type or CD204−/− silica-exposed mice, evidence from other studies suggests that CD204 may play a specific role in the recognition and uptake of apoptotic cells.

CD204 null mice produce elevated TNF-α in response to silica. A wealth of studies has demonstrated the importance of the T helper type 1 (Th1) cytokine TNF-α to the development of inflammation and fibrosis in several murine models of fibrotic lung disease. Therefore, to address whether CD204 is involved in TNF-α production, lavage fluid was collected from untreated and silica-exposed mice and analyzed for TNF-α content. No significant difference was observed in baseline...
TNF-α levels in the lavage fluid between 129Sv wild-type and CD204−/− mice (Fig. 6A). However, following silica exposure, CD204−/− mice exhibit significantly increased production of TNF-α in the lavage fluid at both 4 and 24 h postexposure compared with 129Sv wild-type mice (Fig. 6A). Cell differentials were performed on the lavage cells of the same mice used for TNF-α analysis to examine for the presence of neutrophils indicative of inflammation. As expected, neither 129Sv wild-type nor CD204−/− unexposed mice showed any appreciable presence of neutrophils (Fig. 6B). Interestingly, increases in the percentage of neutrophils present in the lavage fluid were not observed until 24 h postinstillation in either strain of mice, and this response appears to be slightly diminished in CD204−/− silica-exposed mice compared with 129Sv wild-type exposed mice (Fig. 6B).

DISCUSSION

Silicosis is caused by the deposition of silica particles in the lungs of human or experimental animal models resulting in pulmonary inflammation, fibrosis, and carcinogenesis (2, 14, 40). Whereas the anatomical consequences to silica exposure have been well characterized, the molecular mechanisms behind such devastating results remain elusive. The primary objective of this investigation was to elucidate the link between CD204 and the downstream events of inflammation and fibrosis following silica exposure in vivo. CD204−/− AM were shown to recognize and uptake silica in vitro, although this response was attenuated compared with 129Sv wild-type mice. Moreover, following exposure to environmental particles, CD204−/− macrophages exhibited improved cell viability compared with 129Sv wild-type macrophages. In vivo silica exposure resulted in significantly increased production of TNF-α in the lavage fluid of CD204−/− mice compared with 129Sv wild-type mice. Finally, 129Sv wild-type mice displayed typical focal lesions, interstitial thickening with increased connective tissue matrix, and cell infiltrate into air space. In contrast, CD204−/− mice exhibited little to no deposition of collagen yet demonstrated enhanced accumulation of inflammatory cells largely composed of neutrophils. Our findings point to an important role of CD204 in mounting an efficient and appropriately regulated immune response against inhaled particles.

Flow cytometry techniques demonstrated that 129Sv and CD204−/− AM recognize and uptake crystalline silica during a 4-h exposure period in vitro. Although these results demonstrate CD204 is responsible for a specific portion of the recognition and binding of silica particles to AM, they also indicate that other receptors contribute to the recognition and uptake of silica particles. Candidate molecules that may account for this remaining silica uptake include MARCO and CD36, which have previously been shown to also recognize and mediate binding of AcLDL, a ligand of CD204 (17, 68). Further studies are needed to clarify the role other pattern
Environmental particles, macrophages derived from 129Sv wild-type mice were exposed to silica or titanium dioxide at varying concentrations for 4 h. Fluorometric viability assays revealed increased cell injury and decreased survival in response to crystalline silica in 129Sv macrophages, as expected from the published literature. Although CD204−/− macrophages also showed cell injury and decreased survival in response to silica exposure, this response was significantly attenuated compared with 129Sv wild-type macrophages. In contrast, titanium dioxide exerted no effect on cell viability in either wild-type or CD204−/− macrophages at any of the doses examined. Together, these results conclusively illustrate that CD204 is specifically involved in silica recognition and cell susceptibility to silica exposure.

Whereas these results demonstrate that CD204 is involved in silica uptake and cell fate, little data exist regarding the role of CD204 in development of fibrosis in vivo. The fluorescent dye Lucifer yellow selectively stains connective tissue matrix macromolecules, permitting visualization as well as quantification of collagen matrix material using advanced microscopy methods in a manner that yields comparable fibrosis values to the traditional hydroxyproline assay (3, 4, 58). Therefore, LSC represents an attractive alternative to standard hydroxyproline assays as well as an innovative means to rapidly quantify collagen deposition and the development of fibrosis within intact lung sections while immunostaining serial sections for other proteins of interest (3, 4). The combination of Lucifer yellow and LSC demonstrated that 129Sv wild-type mice showed significant collagen deposition and development of fibrosis following silica exposure in 129Sv mice compared with untreated control. In contrast, CD204−/− mice exposed to silica and examined at the same 6-mo postinstillation time point revealed neither collagen deposition nor fibrosis compared with untreated CD204−/− mice. These findings suggest that the development of fibrosis is a very precise process that requires a particular interaction on AM through an as yet unknown interaction with CD204. These data are particularly interesting in light of the concomitant presence of abnormal numbers of inflammatory cells within the lung tissue of CD204−/− silica-exposed mice. Although 129Sv wild-type mice showed areas of focal inflammation following silica exposure, these areas were quite small compared with the extensive infiltration of inflammatory cells observed in CD204−/− mice and suggests a disconnect between the complex processes of inflammation and fibrosis.

The failure of CD204−/− 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 type of response to a Th2 type of response, as has been proposed by a number of recent fibrosis models (28, 31, 34). Consistent with this possibility are studies that implicated CD204 involvement in the regulation of IL-12 release from AM (7, 30). CD204 ligands such as fucoidan are known to induce TNF-α and IL-1β production in macrophage cell lines (11, 24). Furthermore, following endotoxin exposure, CD204−/− mice exhibited significantly higher plasma levels of IL-6 and TNF-α, whereas IL-10 showed no change relative to wild-type mice at 14 days (22). Therefore, interaction of silica with CD204 on AM may promote Th1/Th2 orientation of immune responses by altering cytokine production.

Among the cytokines secreted following phagocytosis of silica particles, TNF-α has been shown to play a critical role in recognition and SR might play in the recognition and binding of silica.

SR mediate physiological and pathological interactions of inhaled particulate matter with AM. Previous work from our laboratory using macrophage cell lines and transfected cells suggested a role for CD204 in the regulation of silica-induced apoptosis in both human and murine cells (9, 20, 27). In particular, a specific role for CD204 in the regulation of silica-induced apoptosis was identified (20), and further study revealed that SR are necessary for caspase activation and subsequent apoptosis (as well as necrosis) caused by silica in a macrophage-like cell line (9). Additional work demonstrated the ability of human AM to undergo apoptosis in response to silica exposure in vitro and that blocking with the SR ligand-binding inhibitor polyinosinic acid ablated this response (27). The hypothesis has been put forward that apoptosis of AM precedes the development of chronic inflammation necessary for progression to lung fibrosis. To assess whether CD204 alters cell viability following interactions with inhaled environmental particles, macrophages derived from 129Sv wild-type and CD204−/− mice were exposed to silica or titanium dioxide at varying concentrations for 4 h. Fluorometric viability assays revealed increased cell injury and decreased survival in response to crystalline silica in 129Sv macrophages, as expected from the published literature. Although CD204−/− macrophages also showed cell injury and decreased survival in response to silica exposure, this response was significantly attenuated compared with 129Sv wild-type macrophages. In contrast, titanium dioxide exerted no effect on cell viability in either wild-type or CD204−/− macrophages at any of the doses examined. Together, these results conclusively illustrate that CD204 is specifically involved in silica recognition and cell susceptibility to silica exposure.

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the pathogenesis of silicosis (15, 42), and actions that antagonize the biological effects of TNF-α ameliorate silica-induced fibrosis in mice (46, 47). TNF-α may also be necessary for the expression of other more downstream growth factors and the consequent development of fibrosis (54, 55). In the present study, TNF-α levels were examined in lavage fluid at 4 and 24 h postinstillation to assess the response of a stereotypical Th1 cytokine to silica exposure in 129Sv wild-type and CD204−/− mice. The data demonstrate a significant increase in TNF-α levels in CD204−/− mice in response to silica, indicative of an enhanced Th1 response.

The pulmonary inflammatory response to inhaled silica involves increases in macrophages and an influx of neutrophils (8, 38); therefore, it was critical to determine the cell type responsible for pulmonary inflammation observed in silica-exposed CD204−/− mice. Immunostaining for the markers F4-80 (macrophages) and NIMP-R14 (neutrophils) demonstrated the majority of inflammatory cells within the lungs of CD204−/− silica-exposed mice were NIMP-R14+ neutrophils and not F4-80+ macrophages. Moreover, 129Sv and CD204−/− mice examined at 1 and 4 wk postexposure of silica showed neither differences in the accumulation of inflammatory cells nor any appreciable increase in collagen deposition in either strain of mice (data not shown), indicating that the accumulation of inflammatory cells in CD204−/− mice occurs over time. These data are consistent with a recent report demonstrating increased infiltration of neutrophils in CD204-deficient mice in response to thioglycollate peritonitis (12). It has been suggested that fibrosis begins as an inflammatory reaction with infiltration of various cell types followed by the enhanced production of cytokines (8, 36). Although the role of neutrophils in pulmonary fibrosis is not clear, studies suggest that neutrophils may also play a modulatory role in the fibrotic process through altered cytokine production (56) and that the duration of neutrophil activation correlates with pulmonary fibrosis (29). The results observed herein using 129Sv and CD204−/− mice indicate that excessive accumulation of inflammatory cells does not necessarily lead to fibrosis and that they are separate events.

Apoptosis or programmed cell death has been reported to play an important role in the resolution of pulmonary inflammation (39, 60). In support of this, recent studies revealed that apoptosis is involved in pulmonary disorders resulting from exposure to bleomycin, silica, and endotoxin, whereas inhibition of apoptosis by gene deletion strategies or by caspase inhibitors blocks the pathological effects of these agents, supporting the role of apoptosis in inflammatory lung disorders (6, 37, 59). Although we have not confirmed the presence of apoptotic cells in the lung tissue of either 129Sv or CD204−/− silica-exposed mice using currently available markers of apoptosis (data not shown), the loss of CD204 on AM may result in inability to adequately recognize and clear apoptotic cells, contributing to the excessive accumulation of inflammatory cells or debris in CD204−/− mice following silica exposure. This hypothesis is supported by studies in which silica-induced inflammation and subsequent apoptosis overwhelmed macrophage capacity to ingest apoptotic neutrophils, resulting in persistent pulmonary inflammation in rats (66). Although the contributions of CD204 to apoptotic clearance in vivo have been difficult to determine and results are contradictory (35, 49, 50), further studies are necessary to clarify the contribution of CD204 to recognition of apoptotic neutrophils in the lungs and the identity of the apoptotic ligand.

In summary, this study indicates that 1) CD204 plays a role in the recognition and uptake of silica, 2) CD204 deficiency attenuates cell death following acute silica exposure, 3) excessive accumulation of inflammatory cells in CD204−/− mice does not necessarily lead to fibrosis and that they are separate events, and 4) CD204−/− mice exhibit significantly increased levels of the cytokine TNF-α in response to silica, which may be indicative of an enhanced Th1 response. Together, these findings provide evidence of an important role of CD204 in mounting an efficient and appropriately regulated immune response against inhaled silica particles.

NOTE ADDED IN PROOF

While our manuscript was under review, preliminary studies were conducted demonstrating the role of CD204−/− mice on the C57Bl/6 background demonstrated enhanced inflammation following silica exposure. In contrast to the results presented here, CD204−/− mice on the C57Bl/6 background exhibited enhanced collagen deposition following silica exposure when measured using the Sircol-soluble collagen assay compared with silica-exposed C57Bl/6 wild-type mice. However, the Sircol assay also demonstrated significantly increased collagen following silica treatment of Gld mice and TNFR1/2 null mice previously reported not to develop fibrosis. The relationship between these results remains to be determined, and current studies are underway to establish the connection between CD204, background strain, and collagen deposition.

GRANTS

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