Scavenger receptors (SR), on the surface of the macrophage, appear to be responsible for silica uptake and cell death signaling in the macrophage. The purpose of this study was to isolate which SRs (macrophage receptor with collagenous structure (MARCO), CD204, or CD36) were involved using a variety of SR single and double null mice. The findings indicated that MARCO was the critical SR involved in silica uptake and cytotoxicity in the primary alveolar macrophages (AM) from C57BL/6 mice, as there was no particle uptake or cell death in the absence of this SR. The level of MARCO expression on AM changed significantly with the absence of other SR, and silica uptake was proportional to cell surface MARCO expression. In addition, silica uptake and cytotoxicity were completely blocked by an anti-mouse MARCO antibody. Transfection of Chinese hamster ovary cells with human MARCO supported these conclusions, as silica particles bound to and initiated apoptosis in the MARCO-transfected cells. Strain differences with regard to SR distribution were also examined. There was a differential expression of these SR on AM from each strain, with MARCO dominant for C57BL/6, CD36 dominant on BALB/c, and all three SR expressed on 129/SvJ mice. Similar to the results with C57BL/6 AM, MARCO was involved with silica-induced cell death in the 129/SvJ strain. In contrast, BALB/c AM used an unidentified mechanism for silica uptake because the SR antibodies failed to block particle internalization. Taken together, these results indicate MARCO is the primary AM receptor interacting with silica, depending on mouse strain and level of constitutive expression.

Inhaled silica particles are a known initiator of several human pathologies including silicosis, autoimmune disorders, and possibly even lung cancer (1, 2). The earliest contact with inhaled silica occurs when the alveolar macrophage (AM) engulfs foreign material. This can lead to cell death in a fraction of the exposed cells, which may be the initial step in the disease process. Scavenger receptors (SR) on the surface of AM are one possible mechanism for the uptake and cell death signaling in the AM (3).

SR are transmembrane glycoproteins found on macrophages, endothelial cells, and smooth muscle cells that bind to a broad range of ligands including Gram-negative bacteria, apoptotic cells, oxidized low-density lipoproteins, and polyinosinic acid to name just a few (4). The SR are divided into several different classes based on structure and function, but they all bind a range of ligands with polyanionic surface characteristics (5). The exact function of SR is still being determined, but they are believed to be an important feature of the innate immune response (6) and they are generally up-regulated in the presence of infection (7). Due to the promiscuous nature of these receptors, multiple functions have been suggested including, but not limited to, endocytosis followed by receptor recycling, cell adhesion (8), transcytosis, intracellular signaling (5), and uptake of particles with polyanionic surface characteristics (9).

Class A SR include macrophage receptor with collagenous structure (MARCO) (10), three splice variants of SR-A (CD204) (5), and two novel members, SRCL (11) and recently discovered SCARA5 (12). CD204 has been shown to be involved with the uptake of titanium dioxide (TiO2), silica, diesel particles, and latex beads in AM (3). In addition, silica-induced apoptosis was demonstrated following the transfection of Chinese hamster ovary (CHO) cells with murine CD204 (13). In this instance, TiO2 was not toxic to the transfected CHO cells indicating a specific toxic response to silica particles, which was inhibited by known antagonists of CD204 (13).

Similarly, MARCO has been identified as the main binding receptor for unopsonized particles and bacteria on human AM using MARCO-transfected COS cells (14, 15), and the presence of MARCO may be the frontline defense for pneumococcal pneumonia, and clearance of inhaled particles (16). Despite the similarity in structure and function between MARCO and CD204, there is evidence that their regulation and signaling properties may be different. This is based on work with MARCO and CD204 null mice, where significant differences in peritoneal macrophage IL-12 production were observed following lipopolysaccharide and IFN-γ stimulation (17). In additional findings, MARCO expression was increased by Th1 polarizing factors and decreased by Th2 polarizing agents. This pattern was reversed for CD204 expression indicating that...
these proteins are differentially regulated (17). Murine strain differences in MARCO and CD204 expression have also been observed, with MARCO being the constitutively expressed SR in the C57BL/6 strain (9, 18). In humans, the expression of MARCO under normal circumstances is relatively low compared with CD204, but it can be induced by a variety of stimulants including the presence of bacteria (17).

Class B SR include CD36, which is known to bind modified proteins (e.g. oxidized low density lipoprotein), and phosphatidylserine (9). CD36 internalizes modified low density lipoproteins in a lipid raft-dependent and caveolin-independent process (19). Similar to CD204, CD36 is one of the main receptors for internalizing oxidized low density lipoprotein in the macrophage cell (20, 21). However, there is no reported evidence that unopsonized particles, such as silica, bind to the CD36 receptor (9).

This study was conducted to determine the relative contribution of these three SR (MARCO, CD204, and CD36) on the uptake and toxicity of silica particles by the murine AM, using single and double null mice on the C57BL/6 and 129/SvJ backgrounds. In addition, murine strain differences in the relative distribution of SR were examined. The primary hypothesis of this work is that MARCO mediates silica uptake and toxicity in the C57BL/6 mouse due to the fact that MARCO is the constitutively expressed SR on the AM. Second, murine strain differences in SR expression are examined to explain differential AM responses to silica particles.

**EXPERIMENTAL PROCEDURES**

**Mice**—C57BL/6, BALB/c, and 129/SvJ mice were obtained from Jackson Laboratories (Bar Harbor, ME). All SR null mice were on a C57BL/6 background. MARCO and MARCO/CD204 null breeder pairs were obtained from Dr. L. Kobzik (Harvard School of Public Health, Boston, MA). CD36 and CD36/CD204 null breeder pairs were obtained from Dr. M. Febbraio (Cleveland Clinic Foundation, Cleveland, OH). Animals were housed in microisolators on a 12-h light/dark cycle. The mice were maintained on an ovalbumin-free diet and given deionized water at libitum. Mice, 7–10 weeks of age, were used for all animal experiments. All animal procedures were approved by the University of Montana Institutional Animal Care and Use Committee (IACUC).

**Particles**—Crystalline silica (min-U-sil-5), 1–5 μm in diameter from Pennsylvania Sand Glass Corp. (Pittsburgh, PA), was acid washed in 1 M HCl at 100 °C. The silica was then washed in sterile water three times and dried in an oven at 200 °C to remove all water. A stock suspension of 2.5 mg/ml in phosphate-buffered saline (PBS) was generated before each experiment. The stock suspension was dispersed by sonic disruption for 1 min prior to use. Titanium dioxide (TiO2) was purchased from Fisher Scientific (catalog number T315-500), and used unprocessed.

**Alveolar Macrophage Isolation and Culture**—Mice were euthanized by a lethal injection of Euthasol™M. The lungs were removed with the heart and then lavaged with five 1.0-ml aliquots of cold PBS. Pooled cells were centrifuged at 400 × g for 5 min. The lavage fluid was aspirated and discarded. The cell pellet was resuspended in 1 ml of RPMI 1640 culture media (Mediatech Inc., Herdon, VA) supplemented with antibiotics and antimycotics (Invitrogen). A 40-μl sample was counted using a Z1 Coulter Particle Counter (Beckman Coulter). The cells were adjusted to 105 per ml and added to 0.65-ml sterile polypropylene tubes at 500 μl/tube. The silica suspensions were added and the cells were cultured in a tumbling suspension culture for 4 h at 37 °C in a water-jacketed CO2 (5%) incubator (ThermoForma, Mariette, OH). In experiments where SR antibodies were used, the antibodies were added (10 μg/ml) 30 min prior to silica addition. The azide-free SR antibodies included anti-mouse MARCO (R & D Systems, Minneapolis, MN), anti-mouse CD204 (Serotec, Raleigh, NC), and anti-mouse CD36 (BD Biosciences).

**Alveolar Macrophage Viability Assay**—Isolated AM were cultured in suspension (106 cells/ml) with varying concentrations of silica for 4 h at 37 °C. At the end of this period, 10 μl of the culture supernatant was removed and mixed with 10 μl of 0.4% trypan blue solution (Sigma). The resulting mixture was added to a hemacytometer and the cells were examined by light microscopy. One hundred random cells were counted per sample, and cells that appeared to contain blue dye were considered dead. Data were expressed as percent of cells excluding trypan blue dye (percent living cells).

**Alveolar Macrophage Apoptosis Assay**—Apoptosis was determined using the Cell Death ELISA (Roche Biochemicals, Indianapolis, IN), and was performed according to the manufacturer’s protocol. Briefly, isolated alveolar macrophages were cultured in suspension (106 cells/ml) with varying concentrations of silica for 4 h at 37 °C. At the end of this period 100 μl of the culture supernatant (105 cells) was removed, washed with PBS, and the resulting cell pellet was lysed with the buffer provided in the kit. The lysate was assayed in ELISA format for histone-bound DNA fragments. The optical density (OD) is read at 405 nm and the background was subtracted out of the final results. Data are expressed as the average OD ± S.E.

**Determination of Scavenger Receptor Expression on Alveolar Macrophages**—Immediately following the AM isolation described above, the cells were exposed to Fc block (1:50) for 30 min at room temperature. Anti-F4-80 phycoerythrin (Caltag, Burlington, CA) and anti-CD11c antigen-presenting cell (APC) (BD Pharmingen, San Jose, CA) were then added to the cells at a 1:50 dilution along with the specific anti-mouse SR antibody (anti-MARCO fluorescein isothiocyanate at a 1:5 dilution (Serotec, Raleigh, NC), anti-CD204 fluorescein isothiocyanate at a 1:50 dilution (Serotec), or anti-CD36 Alexa 488 at a 1–50 dilution (BioLegend, San Diego, CA)) and allowed to incubate for 30 min at room temperature with agitation 2–3 times. Finally, AM were washed twice with PBS and resuspended in 0.5 ml of PBS and analyzed immediately on a FACs Aria flow cytometer using FACS Diva software (BD Biosciences). Flow cytometric methods detected cells that were positive for SR, whereas gating on alveolar macrophages that were dual positive for the markers CD11c and F4–80.

**Quantification of Silica Binding and/or Uptake for Alveolar Macrophages**—Use of flow cytometric side scatter properties to quantify cell/particle binding and uptake is described elsewhere (22). In this study, following silica exposure for 1.5 h, the cells were exposed to Fc block (1:50) for 30 min at room temperature. Anti-F4-80 phycoerythrin (Caltag) and anti-CD11c APC
MARCO Mediates Silica Uptake and Toxicity in the Macrophage

(BD Pharmingen) were added to the cells at a 1:50 dilution and allowed to incubate for 30 min at room temperature with agitation 2–3 times. Finally, AM were washed twice with PBS and resuspended in 0.5 ml of PBS and analyzed immediately on a FACs Aria flow cytometer using FACs Diva software (BD Biosciences). Flow cytometric methods detected silica uptake into the AM by assaying for changes in forward and side scatter properties while gating on AM that were dual positive for markers CD11c and F4-80 (23). All AM retrieved by the lavage technique are positive for both markers. F4-80/CD11c− cells indicates infiltrating monocytes, whereas F4-80+/CD11c+ cells are considered dendritic cells. The later two cell types, if present, were excluded from the analysis. There was no effect of SR deletion on the expression levels of either surface marker (cytometric scatterplots of WT versus MARCO null AM labeling are available in the supplemental data). Data are expressed as average side scatter intensity with arbitrary numerical units.

Lymphocyte Isolation and Culture—OT-II transgenic mice on a C57BL/6 background were euthanized by a lethal injection of Euthasol™ and their spleens were removed and placed in Hank’s buffered saline (Invitrogen) with 2% heat-inactivated fetal bovine serum (Mediatech Inc., Herdon, VA) on ice. The spleens were ground up between two sterile frosted glass slides and filtered through sterile gauze into 50-ml centrifuge tubes. The cell suspension was centrifuged 200 × g for 10 min. The nucleated cell fraction was counted by lysing the red blood cells with Zap-oglobin reagent followed by the Z1 Coulter particle counter (Beckman Coulter, Hialeah, FL). The cell suspension was adjusted to 5 × 10^7 nucleated cells per ml in the media described above and the Spin Sep™ murine T cell enrichment (Stem Cell Biotechnology, Vancouver, BC, Canada) was performed according to the manufacturer’s protocol. The resulting cell recovery was >96% CD 3 positive by FACS analysis. The T cells were suspended in RPMI 1640 culture media (Mediatech Inc., Herdon, VA) supplemented with antibiotics and anticytokitics (Invitrogen) at 2 × 10^6 cells/ml.

Antigen-presenting Cell Assay—Following a 1-h AM suspension culture ± silica (100 μg/ml) or TiO_2 (50 μg/ml), the AM were plated in 96-well tissue culture plates (Costar, Corning, NY) at 5 × 10^4 cells/well and allowed to adhere. Ovalbumin (Sigma) antigen (10 mg/ml), or anti-CD3 antibody (5 μg/ml) was added. This mixture was incubated 2 h at 37 °C in a water-jacketed CO_2 (5%) incubator (ThermoForma, Mariette, OH). The isolated transgenic T cells were added to each well at 2 × 10^5 cells/well. This mixture was incubated 48 h at 37 °C. After 2 days the 96-well plate was centrifuged at 1000 × g for 3 min and the supernatant was retrieved and stored frozen at −20 °C in 0.65 polypropylene tubes until it could be assayed for cytokine levels.

Cytokine Assays—Culture supernatants were assayed for cytokines with commercially available kits according to the manufacturer’s protocol. Interferon γ (IFN-γ) measurements were determined by using Duo-set kits (R & D Systems). Samples were diluted 1:100. Interleukin-13 (IL-13) measurements were determined using Duo-set kits (R & D Systems). Samples were diluted 1:2. Colorometric analysis was performed with the Spectra Max 340 plate reader (GE Healthcare) at 450 nm. Data are expressed as picograms/ml of retrieved culture supernatant.

Transfection of CHO Cells—CHO were cultured in Ham’s F-12 (Mediatech Inc.) with 10% fetal bovine serum plus 100 IU/ml penicillin and 100 μg/ml streptomycin. CHO cells were plated at 1 × 10^6 cells/well in a 6-well cell culture dish overnight and transiently transfected with 4 μg of pcDNA3.1 and hMARCO cDNA (generously provided by Dr. L. Kobzik), using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. The cells were harvested and used after 48 h. The cells were resuspended in PBS containing 0.1% sodium azide and 2% bovine serum albumin, and preincubated with or without primary monoclonal antibody PLK-1 (7 μg/ml) for 15 min on ice. The cells were then treated with TiO_2 (25 μg/ml) or silica (150 μg/ml) and rotated at 37 °C for 30 min, placed on ice, and analyzed by flow cytometry. Binding of particles was measured using the increase in mean side scatter as a marker of increase in granularity of cells due to these particles.

Human MARCO Surface Expression on Transiently Transfected CHO Cells—For immunofluorescence, 1 × 10^6 cells suspended in PBS containing 0.1% sodium azide and 2% bovine serum albumin were preincubated with monoclonal antibody PLK-1 (7 μg/ml) or vehicle at 37 °C for 30 min. The cells were washed twice with PBS containing 0.1% sodium azide and 2% bovine serum albumin. After washing, the antibody PLK-1 was detected using Alexa 488-conjugated goat anti-mouse IgG by flow cytometry. The primary monoclonal antibody PLK-1 (human MARCO) was generously provided by Dr. L. Kobzik.

Apoptosis Detection in MARCO-transfected CHO Cells—Briefly, 5 × 10^5 CHO cells were seeded in 6-well plates and incubated for 24 h. The cells were then transiently transfected as described above. The transfected cells were allowed to recover for 24 h. Cells were then treated with 50 μg/cm^2^ of silica for 7 h at 37 °C. The cells were scraped and centrifuged at 1500 × g for 10 min at 4 °C. The pellets were washed once with 2 ml of PBS, then permeabilized in 1 ml of ice-cold 80% ethanol and left on ice for 1 h. Cells were centrifuged at 1000 × g at 4 °C for 10 min, washed once with 3 ml of ice-cold PBS, and stained with 50 μg/ml propidium iodide in PBS + 0.1% Triton X-100 + 0.1 mM EDTA + 100 μg/ml RNase ON™ at 4 °C. Apoptosis measurement (the sub-G_0/G_1 population) was determined on a FACs Aria Flow cytometer using FACs Diva software (BD Biosciences).

Statistical Analyses—All one-factor experimental designs were analyzed by one-way analysis of variance followed by Dunnett’s comparison to a single control group. All two-factor experimental designs were analyzed by two-way one-way analysis of variance followed by selected Bonferroni’s post hoc pairwise mean comparisons. Pearson’s correlation was used to determine significant associations between factors. Sample size varied between 3 and 9 experimental replications depending on the experiment and desired statistical power. Statistical significance was established as a two-tailed probability of type I error occurring at less than 5%. Analysis and graphics were performed on Prism 4.0 software (GraphPad, San Diego, CA).

RESULTS

To evaluate the activity of different SR in silica-induced cytotoxicity of AM, a variety of SR single and double null mice on the C57BL/6 background were used. The effect of silica on AM
Based on the results in Fig. 1, MARCO surface expression was determined for AM from WT and null mice as described under “Experimental Procedures.” The results are presented in Fig. 2. All of the AM from null mice had a significantly different amount of MARCO surface expression from WT AM. The AM from MARCO−/−, MARCO−/−/CD204−/−, and CD36−/−/CD204−/− mice had significantly lower MARCO expression than WT AM, with the MARCO nulls having no expression above background. However, AM from CD36−/− and CD204−/− mice had significantly increased MARCO expression compared with WT AM.

To further evaluate the cytotoxicity data, AM silica uptake was determined for all WT and null mice as previously described under “Experimental Procedures” using side scatter properties in flow cytometry. These results are illustrated in Fig. 3A. Following a 1.5-h suspension culture at 37 °C, silica (100 μg/ml) produced a significant increase compared with baseline (no particle control) in side scatter for AM from WT mice. Likewise, AM from CD204−/−, CD36−/−, and CD36−/−/CD204−/− mice had similar significant increases in side scatter with silica exposure compared with the corresponding baseline control. In contrast, AM from MARCO−/− and MARCO−/−/CD204−/− mice showed only a slight, nonsignificant increase in side scatter with silica exposure. AM from CD204−/− and CD36−/− showed the highest side scatter in response to silica. The baseline side scatter (no silica) across groups was not significantly different. There was no detectable silica-induced apoptosis or necrosis (potentially confounding the side scatter data) at the end of the 90-min culture (kinetic viability and apoptosis data along with photographic evidence of binding inhibition of silica at 90 min are available in the supplemental data).

Because there appeared to be a correlation between MARCO expression and silica binding, a linear regression plot of.....
MARCO expression against side scatter intensity was generated and can be found in Fig. 3A. The degree of MARCO expression is highly correlated with silica uptake with a significant Pearson’s $r$ of 0.91. Eighty-four percent of the variability in side scatter can be accounted for by the variability in MARCO expression. Therefore, MARCO expression would be a good predictor variable for silica uptake in the C57BL/6 AM model.

Because MARCO appeared to be the best candidate for silica binding and toxicity, AM from both WT and CD36$^{-/-}$ mice were used with an anti-mouse MARCO antibody. The WT represents the baseline response to silica and the CD36$^{-/-}$ AM showed the most toxicity and particle uptake. Briefly, anti-mouse MARCO (azide free, R & D Systems) at 5 g/ml was preincubated with the AM for 30 min prior to silica exposure. The culture lasted an additional 4-h with and without silica (100 g/ml) as described above. These results can be found in Fig. 4A. Fig. 4A shows the effect of anti-MARCO on silica uptake. Significant increases in side scatter with silica exposure were completely reversed in the presence of MARCO antibody. This inhibition of particle uptake was seen in AM from both WT and CD36$^{-/-}$ mice. Likewise, the significant loss of viability seen in the presence of silica (100 g/ml) was also completely inhibited by MARCO antibody as illustrated by Fig. 4B. Fig. 4C shows that silica-induced apoptosis was also significantly attenuated in the presence of MARCO antibody. This is consistent with the observations on viability in the previous figure. Because MARCO antibody was completely effective in preventing silica uptake and toxicity, it can be concluded that CD204 and CD36 do not contribute to silica recognition in C57BL/6 AM.

Having established the importance of MARCO in recognition, binding, and toxicity of silica particles, it was necessary to test the hypothesis that silica modulations to macrophage function would likewise be affected. The silica particle effect on macrophage APC activity is well described elsewhere (24, 25). Fig. 5, A–D, illustrates the lymphocyte-derived cytokines IL-13...
MARCO Mediates Silica Uptake and Toxicity in the Macrophage

A–D, mean ± S.E. ovalbumin-stimulated IL-13 release in 48-h macrophage/lymphocyte co-culture. B, mean ± S.E. anti-CD3-stimulated IL-13 release in 48-h macrophage/lymphocyte co-culture. C, mean ± S.E. ovalbumin-stimulated IFN-γ release in 48-h macrophage/lymphocyte co-culture. D, mean ± S.E. anti-CD3-stimulated IFN-γ release in 48-h macrophage/lymphocyte co-culture. Open bars indicate AM from C57BL/6 wild-type. Black bars indicate AM from MARCO+/−. Dashed line indicates 95% confidence limits of baseline cytokine release. ***, indicates p < 0.001 compared with corresponding wild-type control by Bonferroni’s post hoc test. Sample size n = 3.

FIGURE 5. Alveolar macrophages from MARCO−/− are resistant to silica-induced APC hyper-stimulation of T cells. A, mean ± S.E. ovalbumin (OVA)-stimulated IL-13 release in 48-h macrophage/lymphocyte co-culture. B, mean ± S.E. anti-CD3-stimulated IL-13 release in 48-h macrophage/lymphocyte co-culture. C, mean ± S.E. ovalbumin-stimulated IFN-γ release in 48-h macrophage/lymphocyte co-culture. D, mean ± S.E. anti-CD3-stimulated IFN-γ release in 48-h macrophage/lymphocyte co-culture. Open bars indicate AM from C57BL/6 wild-type. Black bars indicate AM from MARCO+/−. Dashed line indicates 95% confidence limits of baseline cytokine release. ***, indicates p < 0.001 compared with corresponding wild-type control by Bonferroni’s post hoc test. Sample size n = 3.

and IFN-γ resulting from antigen-dependent (ovalbumin-stimulated) and antigen-independent (anti-CD3-stimulated) macrophage/lymphocyte co-cultures. The silica-induced APC hyper-response is evident in all 4 graphs (Fig. 5, A–D). This effect was specific to silica, as TiO₂ did not stimulate cytokine release significantly above control. In addition, the silica-stimulated MARCO−/− AM co-cultures had significant reductions in T cell cytokine release compared with wild-type regardless of the stimulant (ovalbumin or CD3 antibody). The difference in Fig. 5C failed to reach statistical significance, although it does represent a 50% reduction in IFN-γ release relative to control. The absence of MARCO appeared to have functional consequences with regard to the behavior of AM exposed to silica particles, suggesting that silica binding, internalization, and/or apoptosis are necessary for the increase in APC activity. However, the internalization of silica may be the most important factor as MARCO null AM still had a residual increase in APC activity, possibly due to a non-receptor-mediated endocytosis.

To further evaluate the role of MARCO in silica binding, CHO cells were transfected with human MARCO (hMARCO) as described under “Experimental Procedures.” The resulting transfected cells were exposed to silica (150 μg/ml) and TiO₂ (25 μg/ml) for 30 min in suspension culture at 37 °C. The relative change in side scatter between empty vector transfection controls and hMARCO-transfected cells is found in Fig. 6A. Presence of hMARCO on CHO cells clearly increased side scatter with the addition of particles (both TiO₂ and silica). Furthermore, with regard to silica, binding was blocked by preaddition of anti-hMARCO (PLK-1) antibody also shown in Fig. 6A. Fig. 6B shows significant increases in apoptosis (increases in sub-G₀/G₁ cell population by propidium iodide cell cycle assay) when hMARCO-transfected CHO cells were exposed to silica particles. This effect was partially inhibited by preincubation with anti-hMARCO (PLK-1) antibody (data not shown).

Although MARCO appears to be the SR responsible for silica binding and toxicity in the C57BL/6 strain, it was not clear if that was the case for other mouse strains. Fig. 7 shows the constitutive AM expression levels of MARCO, CD204, and CD36 for three different WT mouse strains. Freshly isolated AM were stained for surface markers as described under “Experimental Procedures.” The C57BL/6 AM were characterized by dominant MARCO expression with some CD36 expression. Unfortunately, CD204 expression cannot be determined in this mouse strain due to a polymorphism in that gene, which alters the antigen preventing the 2F-8 monoclonal antibody from binding (26). In contrast, BALB/c AM had no constitutive MARCO, or CD204 expression, combined with moderate CD36 expression. Last, 129/SvJ AM had all three SR constitutively expressed, with MARCO and CD36 at relatively high levels. These results indicate that the constitutive expression levels
of SR on AM were strain dependent with variations in the levels of expression for any given SR on any given strain.

Because AM from BALB/c mice did not express either MARCO or CD204, it was anticipated that neither would contribute to silica binding and cytotoxicity. BALB/c AM were isolated and treated with silica as described under “Experimental Procedures.” The BALB/c AM were exposed to silica (100 μg/ml) for 1.5 h in the presence or absence of anti-mouse MARCO (5 μg/ml) or 2F-8 antibody (5 μg/ml). The resulting side scatter data are presented in Fig. 8. Similar to AM from the C57BL/6, the AM from BALB/c showed increased side scatter in the presence of silica indicating particle uptake. However, in this case, the MARCO and CD204 antibodies had no effect on this increase indicating little or no role for these receptors in the uptake of silica in BALB/c AM. In addition, using anti-CD36 antibody or the broad-spectrum SR inhibitor polyinosinic acid had no effect on silica uptake, eliminating CD36 and several other SR as possible candidates for silica binding in the BALB/c AM (data not shown).

In addition, there was no cytotoxicity observed when exposing BALB/c AM to 100 μg/ml silica for 4 h (data not shown). The concentration of silica had to be increased to 150 μg/ml before any loss in viability was detected and even then, no significant apoptosis was detected (data not shown). Whereas silica can cause cytotoxicity in BALB/c AM, it is possible that SR of the A class (specifically MARCO) are necessary for apoptosis. In addition, MARCO and CD204 antibodies had no modulating effects on cytotoxicity at this concentration of silica in the BALB/c model (data not shown).

Because the 129/SvJ AM was the only strain to have all 3 SR expressed on the cell surface, it was the best model to determine whether CD204 played a role in silica uptake and cytotoxicity. The anti-mouse SR antibodies were used to selectively block the 3 receptors prior to silica exposure as described under “Experimental Procedures.” Fig. 9A illustrates the silica uptake
MARCO Mediates Silica Uptake and Toxicity in the Macrophage

in 129/SvJ AM. Silica uptake is much greater in this strain when compared with C57BL/6 and BALB/c AM under the same conditions. Similar to the C57BL/6 strain, the anti-mouse MARCO completely blocked particle uptake. The CD204 antibody had no effect. The AM viability results at 4 h (Fig. 9B) are consistent with the particle uptake results above, where anti-MARCO is the only antibody that blocks the silica-induced loss in AM cell viability. Similar to this result, Fig. 9C shows that silica-induced AM apoptosis is also blocked by the anti-mouse MARCO. Anti-mouse CD36 was used in all experiments, but there was no effect similar to anti-mouse CD204 (data not shown). Taken together, it appears that the 129/SvJ AM are similar to the C57BL/6 AM with regard to silica processing. Both strains use constitutively expressed MARCO exclusively to bind and internalize the silica particles, which is in contrast to the BALB/c AM where a less toxic unidentified mechanism was used for silica uptake.

DISCUSSION

The thesis of this work is that the AM MARCO receptor is solely responsible for silica uptake and toxicity in the C57BL/6 mouse model, due to the fact that it is the primary constitutively expressed member of the SR-A family (of the ones examined). The relative contributions of two other SR were examined. There is clearly a common function between MARCO and CD204 based on the results of several studies (3, 9, 14). This is an example of “convergent evolution” where proteins from different genes develop a similar response pattern and function (5). CD36 has some overlapping functions with MARCO and CD204 (e.g. modified low density lipoprotein uptake), but these common functions do not appear to be relevant with regard to silica or particle uptake and toxicity (9).

The silica cytotoxicity studies (Fig. 1) demonstrated that the absence of MARCO was critical to blocking the cytotoxicity of silica. Only AM from the MARCO<sup>−/−</sup> mice and MARCO<sup>−/−</sup>/SRA<sup>−/−</sup> mice were completely resistant to silica-induced cytotoxicity. The absence of CD36 did result in increased loss of viability, but that can be explained by the increase in MARCO expression in those null strains. The absence of CD36 did not lead to a significant increase in apoptosis, however. Interestingly, the absence of CD204 did not have an effect in the C57BL/6 model. This could be explained by the possibility that CD204 is not constitutively expressed on unstimulated C57BL/6 AM. The gene deletion, in this case, would be of no consequence. The only other possible explanation is that the polymorphism in C57BL/6 CD204 results in a nonfunctional receptor with regard to the initial binding of silica. There is ample evidence that CD204 interacts with silica particles initiating apoptosis in AM and other cell models (13, 27). In addition, a recent publication shows that CD204 is important in binding bacteria and environmental particles in an in vivo murine model (28).

The relative distribution of MARCO in the various null strains shown in Fig. 2 suggests that the absence of CD204 or CD36 may influence the expression level of MARCO. Significant differences in MARCO expression (elevations with CD204<sup>−/−</sup> and CD36<sup>−/−</sup>, decreases with CD204<sup>−/−</sup>/CD36<sup>−/−</sup>) were observed when CD204, CD36, or both genes were absent. This might indicate a compensatory mechanism with regard to SR expression due to the various overlapping functions of the SR. Regardless of strain, a relatively small percentage of AM express MARCO constitutively (Fig. 2). This could be due to the maturation state of the AM, with MARCO expression higher in more mature AM. This needs to be determined in future experiments.

The result of the silica uptake study (Fig. 3A) was consistent with the results presented above on silica cytotoxicity. The absence of MARCO, regardless of single or double null model, resulted in a significant reduction for AM silica uptake. However, it did not completely eliminate silica uptake. There was still some silica uptake in the MARCO null models, indicating that a possible alternative process for taking up silica may exist on these AM cells. This unspecified mechanism accounts for roughly 50% of the side scatter increase associated with particle internalization. There are probably two distinct ways silica can be internalized in the AM; one receptor (MARCO)-mediated and potentially cytotoxic, and the other a non-receptor fluid phase endocytosis that is not cytotoxic. Nevertheless, the level of MARCO expression was highly correlated with the amount of silica uptake (Fig. 3B) in the different null models, indicating the receptor-mediated mechanism was dominant in the C57BL/6 model. In contrast, the cytotoxicity measures were not significantly correlated to MARCO expression, regardless of which measure was used (viability or apoptosis). This may indicate that silica cytotoxicity is a more complex phenomenon than the particle uptake. Not much is known about intracellular MARCO signaling, but studies on CD204 ligand binding reveal activation of phospholipase C-δ1, phosphatidylinositol 3-kinase, protein kinase C (29), heterotrimeric G proteins (30), mitogen-activated protein kinases (31, 32), and caspases (27). Due to the similarity in structure and function between MARCO and CD204, it seems reasonable that MARCO could initiate similar intracellular responses.

Further evidence that MARCO is solely responsible for silica uptake and toxicity in AM from C57BL/6 mice is found in Figs. 4 and 6. Use of anti-mouse MARCO completely inhibited particle uptake and toxicity in AM from both WT and CD36<sup>−/−</sup> mice. This further supports the observation that CD36 is not involved in the process. The transfection of hMARCO into CHO cells resulting in silica and TiO<sub>2</sub> binding that was reversed by anti-hMARCO is further proof-of-principle that MARCO is capable of particle binding and uptake. This also demonstrates that silica binding with the MARCO receptor is not an artifact of using the C57BL/6 model indicating relevance to the human condition.

Examination of relative SR expression in the three different mouse strains (Fig. 7) indicates that constitutive SR expression is strain specific. MARCO is the dominant SR only in the C57BL/6 AM model. Based on the previous results (no effect of CD204<sup>−/−</sup>), it is reasonable to assume that CD204 is probably not constitutively expressed in this WT model. The BALB/c AM has no constitutive MARCO or CD204, but it does have CD36. The 129/SvJ AM have all three SR constitutively expressed. Considering the variation between murine strains, the variance in SR expression in humans could be similarly extreme.
MARCO Mediates Silica Uptake and Toxicity in the Macrophage

Consistent with the differential SR expression, Figs. 8 and 9 demonstrate how constitutive SR expression on AM effects silica uptake and toxicity. In the 129/SvJ mouse model, MARCO has a dominant role in silica processing, much like the C57BL/6 model. In the BALB/c model, anti-MARCO and anti-CD204 had no effect on the uptake of silica. This is consistent with the observation that BALB/c had no constitutive expression of either receptor. It also suggests that there is an alternative mechanism for silica uptake in the BALB/c AM. It could be another SR (although polyinosinic acid had no blocking effect in this model) or some unidentified receptor or process. The lack of silica toxicity in the BALB/c model argues for a receptor-mediated cell death mechanism with regard to silica. The silica particles are still being taken up by the AM in the absence of toxicity, suggesting that oxidative stress accompanied with phagocytosis is probably not the cause of cell death as others have implied (33).

In conclusion, the C57BL/6 AM used MARCO for uptake and processing of silica particles. In a percentage of the AM population, this leads to apoptosis and cell death depending on the amount of silica encountered by the cells. This scenario is different in other murine strains because of different constitutive surface receptors responsible for processing of silica. There are probably multiple mechanisms at work in the human lung with regard to the processing of inhaled silica, depending on the constitutive expression of these scavenger receptors for any given exposed individual. It may partially explain the variable susceptibilities to silica exposure and variable resulting pathologies in the human.

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