The Macrophage Scavenger Receptor SR-AI/II and Lung Defense against Pneumococci and Particles

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The class A macrophage scavenger receptor SR-AI/II is implicated as a pattern recognition receptor for innate immunity, but its functional role in lung defense has not been studied. We used mice genetically deficient in SR-AI/II and their wild-type C57BL/6 counterparts to investigate the contribution of this receptor to defense against pneumococcal infection and inhaled particles. SR-AI/II deficiency caused impaired phagocytosis of fluorescent bacteria *in vivo*, diminished clearance of live bacteria from the lungs, and substantially increased pneumonic inflammation. Survival studies also showed increased mortality in SR-AI/II-deficient mice with pneumococcal lung infection. Similarly, after challenge of the airways with TiO₂ particles, SR-AI/II-deficient mice showed increased proinflammatory cytokine levels in lung lavage fluid and a more pronounced neutrophilic inflammation. The data indicate that the lung macrophage class A scavenger receptor SR-AI/II contributes to innate defense against bacteria and inhaled particles.

Keywords: environmental particles; lung; macrophages; scavenger receptors

Macrophages recognize pathogens through a rich repertoire of receptors expressed on their surface. These receptors constitute a large family of pattern recognition receptors, which can be subdivided based on their structure and function. The class A scavenger receptors (SR-As) are a small group of pattern recognition receptors composed of four members: SR-AI/II, MARCO (Macrophage Receptor with Collagenous structure) (1, 2), and the recently identified SRCL (scavenger receptor with C-type lectin) (3) and SCARA5 receptor (4). SR-AI/II is expressed primarily by macrophages and has been extensively studied in the context of atherosclerosis (5–8), where it was initially identified as the macrophage receptor for oxidatively modified lipoproteins (9, 10).

SR-AI/II has been reported to participate in other important biologic processes (11, 12), such as macrophage–host cell interactions (9, 13), endocytosis of modified proteins and ligands (9), phagocytosis of apoptotic cells (14) and microbes (15), and clearance of microbial products, such as LPS (16) and lipoteichoic acid (17). In addition, SR-AI/II engagement by its ligands may be involved in intracellular signaling events in the macrophage (18–22).

SR-AI/II has also been implicated in innate immune responses to systemic infections (6, 15–17, 23–26), as has been demonstrated in mouse models using intravenous or intraperitoneal challenges. For example, SR-AI/II-deficient mice display impaired protection against intravenous infection by *Listeria*

monocytogenes and HSV-1 virus (6) and increased sensitivity to intraperitoneally administered *Staphylococcus aureus* (23). These observations, and our previous finding that the class A receptor MARCO contributes significantly to lung host defense (27), and the expression of SR-AI/II on lung macrophages (28) suggest a potential function for SR-AI/II in lung host defense.

The studies reported in this article tested this postulate using mice genetically deficient in SR-AI/II and receiving pulmonary challenges with pneumococcal bacteria and environmental particles.

MATERIALS AND METHODS

Animals

Male or female mice genetically deficient in SR-AI/II (SR-AI/II^{−/−}) were used in all experiments. SR-AI/II^{−/−} mice were generated by disrupting exon 4 of the SR-A gene, which is essential for the formation of functional trimeric receptors (6). Age- and sex-matched wild-type (C57BL/6 SR-AI/II^{+/+}) mice purchased from Charles River Laboratories (Wilmington, MA) were used as controls. All animals were housed in sterile microisolator cages and had no evidence of spontaneous infection. All experimentation involving animals was approved by the governing institutional review panel.

Reagents and Particles

Titanium dioxide (TiO₂) particles (median diameter ~ 1 μm) were generously provided by Dr. J. Brain (Harvard School of Public Health, Boston, MA). The particles were baked at 200°C for 4 h to destroy any contaminating endotoxins. All particles were suspended in BSS– (124 mM NaCl, 5.8 mM KCl, 10 mM dextrose, and 20 mM HEPES) as stock solution and sonicated before use. All reagents not otherwise specified were obtained from Sigma Chemical Co. (St. Louis, MO).

Bacteria

Streptococcus pneumoniae serotype 3 strain ATCC 6303 (American Type Culture Collection, Rockville, MD) was cultured overnight on 5% sheep blood-supplemented agar Petri dishes (VWR, West Chester, PA). A stock suspension was prepared, aliquoted in small volumes, and kept at –80°C. For each experiment, an aliquot was grown overnight on a blood agar plate and resuspended in sterile isotonic saline. Bacterial concentration of the obtained suspension was estimated by OD₆₀₀ measurements, and the appropriate dilution was prepared in saline to be administered intranasally to mice. The true concentration was determined by overnight culture.

Mouse Model of Pneumococcal Pneumonia

A well characterized mouse model of pneumococcal pneumonia (27) was used in this study. Pneumonia was induced by intranasal instillation of 25 μl of a bacterial suspension containing ~ 10⁵ CFU of *S. pneumoniae* type 3 of mice under short-term anesthesia with halothane. Four or 24 h postinfection, mice were killed with an intraperitoneal injection of a lethal dose of sodium pentobarbital (FatalPlus; Vortech Pharmaceuticals, Dearborn, MI). Separate experiments were performed for bronchoalveolar lavage (BAL) and lung harvest for histology or total lung bacteria counts (CFU). At 4 or 24 h after infection, whole lungs were harvested and homogenized in 1 ml sterile isotonic saline with a tissue homogenizer (Omni International, Warrenton, VA). Serial 10-fold dilutions in sterile saline were made from these homogenates, and 100 μl volumes were plated onto sheep-blood agar plates and incubated at 37°C. CFUs were counted after 18–20 h. In some experiments, to

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assess survival, mice were instilled intranasally with a single 25- μ l suspension of *S. pneumoniae* ($\sim 4.5 \times 10^5$ CFU). Animal survival was monitored twice a day over a period of 13 d.

BAL of Infected Mice

Animals were killed 4 or 24 h after bacterial challenge. BAL was performed *in situ* with a 20-gauge catheter inserted into the proximal trachea, flushing the lower airways five times with 0.8 ml of PBS. The fluid retrieved from the first lavage was used for ELISA assays. The BAL cells were separated from the BAL fluid (BALF) by centrifugation, resuspended in PBS, and counted, and a fraction was cytospun on microscopic slides for staining with Diff-Quick (Baxter Scientific Products, McGaw Park, IL) and for subsequent differential counts.

Preparation of FITC-Labeled Pneumococci and Phagocytosis Assay

S. pneumoniae were labeled with FITC (Molecular Probes, Eugene, OR). Bacteria were grown overnight on sheep blood agar plates, washed twice, and resuspended in PBS. Bacterial concentration was set to $\sim 10^9$ CFU/ml as determined by OD₆₀₀. Bacteria were heat killed at 95°C for 10 min, centrifuged, and resuspended in 1 ml of labeling buffer (0.1 M NaHCO₃, pH 9.2). Newly prepared FITC in DMSO (0.25 mg/ml of suspension) was added, and bacteria were incubated under constant stirring in the dark at room temperature for 1 h. Bacteria were washed three times and dialyzed overnight against PBS. Cell number was determined using a hemocytometer under a fluorescence microscope. The final concentration was set to 10^9 bacteria/ml.

Mice received intranasally 10^7 FITC-*S. pneumoniae* under light halothane anesthesia. Lungs were lavaged with PBS 3 h later, and the amounts of bacteria associated with the alveolar macrophages (AMs) in the BALF were quantified by flow cytometry.

Particle Effects *In Vivo*

Mice were lightly anaesthetized with halothane, and solutions of particles in pyrogen-free saline were administered via intranasal insufflation (50 μ l). Pilot experiments in normal wild-type mice showed minimal response to 1 mg/ml and 5 mg/ml of the inert particle TiO₂. These concentrations were used in subsequent experiments comparing responses of SR-AI/II-deficient mice. Mice were killed 4 h after instillation, and BAL was performed. Total cell yield was determined by hemocytometer and differential by microscopic evaluation of cytocentrifuge preparations stained with modified Wright-Giemsa. Aliquots of cell-free BALF were stored at -70°C for subsequent ELISA assay of cytokines. In some experiments, whole lung tissue was harvested without lavage for RNA extractions using the Trizol reagent (GibcoBRL, Gaithersburg, MD) according to manufacturer's protocol. Expression of a panel of cytokine mRNAs (RANTES, Eotaxin, MIP-1a, MIP-1b, MIP-2, IP-10, MCP-1, and TCA-3) was evaluated using the RNase protection assay kit (mCK5; BD Biosciences, San Jose, CA) following the manufacturer's protocol. Blots were visualized using an InstantImager Phosphorimager (Packard, Downers Grove, IL), and densitometric analysis was performed using the dedicated Imager software package.

Quantitation of BALF Cytokine Content by ELISA

MIP-2 and TNF- α were quantitated in BALF of infected/TiO₂-treated SR-AI/II^{-/-} and control mice using commercially available ELISA kits (R&D Systems Inc., Minneapolis, MN) following the manufacturer's instructions.

Statistical Analysis

Data were analyzed using the StatView software (Abacus Concepts, Berkeley, CA). Differences in survival were determined by χ^2 analysis. Kaplan-Meier curves were used to show survival over time, and differences between curves were analyzed using a log-rank test. For other measurements, differences between groups were examined by Student's *t* test or ANOVA with correction for multiple groups. Data are presented as mean $\pm$ SEM. Results were considered significant at $P \leq 0.05$.

RESULTS

Increased Susceptibility to Lethal Pneumococcal Infection in SR-AI/II^{-/-} Mice

We first tested whether the lack of SR-AI/II would affect survival after lung infection with *S. pneumoniae*. We inoculated SR-AI/II^{-/-} and SR-AI/II^{+/+} mice with 4.5×10^5 CFU of *S. pneumoniae* and monitored their survival over 2 wk. In the wild-type mice group, we observed 40% mortality, with deaths occurring between Days 4 and 6 after infection. SR-AI/II^{-/-} mice showed a much greater mortality at about this same time point, with 50% mortality at Day 5 postinfection and an additional 40% at Day 6, resulting in a total mortality of 90%. No subsequent deaths were recorded in either group. The difference in survival between the two groups of mice was statistically significant (Figure 1).

Impaired Bacterial Clearance in the Lungs of Infected SR-AI/II^{-/-} Mice

We investigated whether the increased vulnerability of the SR-AI/II^{-/-} mice to pneumococcal infection derives from defects in bacterial clearance from the lungs of infected mice. We inoculated the animals with live *S. pneumoniae* and determined total CFU in lung homogenates after 4 and 24 h. The viable bacteria recovered from the lungs of infected SR-AI/II^{+/+} male mice 4 h after infection represented only $8.31 \pm 3.37\%$ of the inoculum, whereas $47.8 \pm 10.12\%$ of the inoculated bacteria were recovered from the lungs of SR-AI/II^{-/-} mice (Figure 2A). We observed differences in bacterial clearance in male and female mice; therefore, the data are presented separately. In female mice, recovered viable bacteria represented $4.9 \pm 1.2\%$ and $23.54 \pm 6.06\%$ of the input in SR-AI/II^{+/+} and SR-AI/II^{-/-}, respectively (Figure 2B). Similar results showing decreased bacterial clearance in the lungs of SR-AI/II^{-/-} mice were seen at 24 h postinfection (Figure 2C).

SR-AI/II^{-/-} Mice Show Increased Pulmonary Inflammation in Response to Pneumococci

Streptococcal pneumonia is characterized by an intense production of proinflammatory cytokines and chemokines by activated AMs and other lung cells. Consequently, large numbers of neutrophils are recruited from the circulation into the alveoli (29, 30). We examined these early features of host defense to determine the consequences of SR-AI/II deficiency on the inflammatory response to pneumococcal infection.

Four hours postinoculum, SR-AI/II^{-/-} mice showed markedly increased BAL cellularity compared with wild-type control mice (e.g., total cells $\times 10^4$: 262 ± 28.5 versus 145 ± 6.45 , respectively, in one representative experiment). Similarly, deficient mice showed more neutrophils ($14.93 \pm 3.23\%$ of total BAL

Figure 1. Absence of SR-AI/II increases mortality in *S. pneumoniae*-infected mice. SR-AI/II^{+/+} and SR-AI/II^{-/-} mice were intranasally inoculated with 4.5×10^5 CFU of *S. pneumoniae* serotype 3, and survival was monitored over 13 d. Data are expressed as the percentage of mice surviving at each time point. Kaplan-Meier survival plots are shown for 10 animals per group. Mantel-Cox logrank test showed a *P* value of 0.0416.

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Figure 2. Diminished bacterial clearance from the airways of SR-AI/II^{-/-} after infection with *S. pneumoniae*. SR-AI/II^{+/+} and SR-AI/II^{-/-} ($n > 6$) were intranasally infected with $\sim 10^5$ CFU of *S. pneumoniae*. Lung bacterial load was determined at 4 h (A) and 24 h (B) postinfection. Data shown are representative of at least three separate experiments and are presented as mean $\pm$ SEM.

leukocytes) than did the wild-type animals ($2.45 \pm 0.46\%$ of total BAL leukocytes) (Figure 3A).

Consistent with the elevated neutrophilic inflammation, BALF from infected SR-AI/II^{-/-} mice showed higher amounts of the cytokines TNF- α and MIP-2 (0.75 ± 0.1 ng/ml and 6.46 ± 0.18 ng/ml, respectively) than did SR-AI/II^{+/+} mice (0.13 ± 0.01 ng/ml and 4.48 ± 0.39 ng/ml, respectively) (Figure 3B).

Diminished Phagocytosis of *S. pneumoniae* in SR-AI/II^{-/-} AMs

To directly evaluate the contribution of SR-AI/II to the phagocytic activity of macrophages within the alveolar space, we tested AM uptake of FITC-labeled *S. pneumoniae in situ*. After intranasal administration of bacteria into wild-type and SR-AI/II-deficient mice, AMs were harvested by lavage 3 h later and analyzed by flow cytometry to measure uptake of fluorescent bacteria. The total fluorescence intensity, reflecting the total number of bacteria, associated with AMs is significantly higher in the wild-type mice than in the deficient mice (304 ± 5 and 232 ± 20 MFI units, respectively) (Figure 4).

SR-AI/II Modulates TiO₂-Induced Inflammation in the Lungs

AMs are also responsible for ingestion and clearance of inhaled environmental particles (31, 32), as can be modeled with the inert particulate TiO₂ (27, 33–37). We sought to test the potential role of SR-AI/II in this process *in vivo* by administering TiO₂ particles intranasally to mice and assessing the inflammatory response in the lower airways. All mice showed similar total leukocyte counts in the BALF after exposure to PBS and TiO₂ (data not shown). However, SR-AI/II-deficient mice exhibited a higher influx of PMNs into the airways in response to 50 μ g TiO₂ ($157 \pm 51 \times 10^3$ PMNs/ml) and 250 μ g TiO₂ ($400 \pm 108 \times 10^3$ PMNs/ml) when compared with wild type-mice ($30 \pm 21 \times$

Figure 4. Decreased phagocytic capacity in SR-AI/II^{-/-} AMs. Mice were intranasally given 10^7 FITC-*S. pneumoniae* under slight anesthesia. Lungs were lavaged with PBS 3 h later, and the amount of bacteria associated with AMs was quantified by flow cytometry. Data shown are pooled from two separate experiments representing a total of seven mice per group.

10^3 PMNs/ml and $191 \pm 83 \times 10^3$ PMNs/ml in response to 50 μ g and 250 μ g TiO₂, respectively) (Figure 5A).

The levels of TNF- α secreted into the BALF from SR-AI/II^{+/+} mice in response to 50 μ g TiO₂ (0.314 ± 0.177 ng/ml) and 250 μ g TiO₂ (0.081 ± 0.084 ng/ml) were not significantly different from the amount found in mice exposed to PBS (0.127 ± 0.022 ng/ml). In contrast, BALF from SR-AI/II^{-/-} contained higher amounts of TNF- α in response to 50 μ g TiO₂ and 250 μ g TiO₂ (0.828 ± 0.348 and 1.613 ± 0.349 ng/ml, respectively) than in response to PBS (0.097 ± 0.032 ng/ml) (Figure 5B). SR-AI/II^{-/-} mice were more responsive to both doses of TiO₂ than were their respective control mice.

Similarly, exposure to TiO₂ induced a higher rate of MIP-2 mRNA expression in the lung tissue from SR-AI/II^{-/-} than from SR-AI/II^{+/+} mice as assessed by RPA (Figure 5C). Overall, TiO₂ treatment induced a 3.45 ± 0.7 -fold increase in MIP-2 expression over PBS treatment in SR-AI/II^{-/-}, compared with a 0.56 ± 0.05 -fold increase in wild-type mice (Figure 5D).

DISCUSSION

Our data support a role of the class A scavenger receptor SR-AI/II on AMs in the innate immune response to bacterial pneumonia and inhaled environmental particles. SR-AI/II^{-/-} mice show defective bacterial clearance, with increased neutrophil and cytokine responses and diminished survival. Similarly, SR-AI/II^{-/-} mice show increased lung inflammatory responses to an “inert” particle, TiO₂. These findings indicate that this class A scavenger receptor contributes substantially to innate defense of the lung in the murine models studied.

Certain aspects of the infection model we used merit discussion. We chose to study infection by *S. pneumoniae* in large

Figure 3. SR-AI/II^{-/-} mice with pneumococcal pneumonia develop a stronger inflammatory response in the airways. BALF was harvested from wild-type ($n = 10$) and deficient mice ($n = 8$) 4 h postinfection, and the total number of leukocytes and the percentage of PMNs were determined (A). MIP-2 and TNF- α contents in the BALF were determined using ELISA (B). Data shown are representative of at least three separate experiments and are presented as mean $\pm$ SEM.

Figure 5. Elevated lung inflammation in SR-AI/II-deficient mice in response to inhaled TiO₂ particles. (A) PMN recruitment into BALF of PBS and TiO₂-treated mice ($n = 3$ –35 mice/group). (B) BALF TNF- α after instillation with PBS or two different doses of TiO₂, as measured by ELISA ($n = 3$ –9 mice/group). (C) Analysis of lung gene expression in the lungs of PBS or TiO₂-treated mice by RNase protection assay shows increased MIP-2 response in SRA-I/II-deficient mice. (D) Fold-increase of MIP-2 mRNA expression in TiO₂-treated mice over PBS-treated mice in SR-AI/II^{+/+} ($n = 3$) and SR-AI/II^{-/-} mice ($n = 4$).

part because it is the most common causative microorganism in community-acquired pneumonia (38, 39). We also used a relatively low-dose challenge, aiming to model the frequent low-dose challenge to the lungs of bacteria within aspirated nasopharyngeal secretions. The pathogenesis of pneumococcal pneumonia in most cases involves initial colonization of the upper airways. Pneumococcal infection is relatively common but is far less common than might be predicted from high rates of nasopharyngeal colonization in normal adults and children (40) and from the frequency of normal nocturnal aspiration of upper airway secretions (41). These data imply that lung macrophage uptake and clearance can provide effective innate defense against these low-dose inocula, and our experimental design sought to analyze the role of macrophage SR-AI/II in this process.

SR-AI/II^{-/-} mice showed only partial impairment of *in vivo* uptake of fluorescent *S. pneumoniae*. The deficiencies in clearance observed using live bacteria were more profound, and one possible explanation is that the smaller effect observed with fluorescent bacteria may reflect changes induced by the heat-killing process used to prepare the bacteria for labeling. Nevertheless, these data are qualitatively consistent with diminished *in vivo* uptake of bacteria by lung macrophages in SR-AI/II-deficient mice.

Prior studies have found that SR-AI/II protects against systemic infections by gram-positive and gram-negative bacteria. Our data expand the role of SR-AI/II to protection of the lungs against pneumococcal infection, a major cause of community-acquired pneumonia (42). Our present findings, together with previously published work (27), suggest that SR-AI/II and another class scavenger receptor, MARCO, may act together to prevent the onset of pneumococcal pneumonia after aspiration of *S. pneumoniae* from the nasopharynx, providing an explanation for the low percentage of pneumococcal infections when compared with the total number of people carrying pneumococcus (41, 43). SR-AI/II-deficient mice show increased basal expression of MARCO on their normal AMs (A. Holian, personal communication). The questions of whether and how much this

increased expression contributes to the residual clearance capacity seen in our experiments remain unanswered. The use of mice lacking MARCO and SR-AI/II may further inform assessment of the relative contribution to innate defense by these two receptors.

Another challenge to innate defenses of the lung is air pollution. Chronic exposure to particulate air pollution has been associated with various adverse health effects in humans (44, 45). Absence of SR-AI/II was associated with markedly increased neutrophilic inflammation after TiO₂ challenge, likely mediated by the increased expression of MIP-2, a potent neutrophil chemoattractant cytokine. These findings prompt the speculation that the enhanced effects of TiO₂ in SR-AI/II-deficient mice may result from prolonged contact of particles with other cell types of the lungs, such as the epithelial cells, leading to MIP-2 synthesis and subsequent recruitment of neutrophils (27).

A final question to consider is the relevance of these data using mice to human biology. Although SR-AI/II is expressed on normal human AMs, we have recently observed a dominant role for MARCO in human AM scavenger receptor functions tested *in vitro*. Blocking antibodies to human SR-AI/II did not affect human AM binding of fluorescent bacteria or TiO₂ (33). This raises the possibility that the observations reported here using murine models may not apply to human biology. However, the *in vitro* studies of normal human AMs have not addressed the potential requirement for surfactant components to optimize SR-AI/II function *in vivo* (46) or the potential for increased function after modulation of scavenger receptor expression by cytokines or other components of inflammation (47, 48). This remains a question to be addressed in future studies.

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

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