The Scavenger Receptor MARCO Is Required for Lung Defense against Pneumococcal Pneumonia and Inhaled Particles

Mohamed Arredouani,¹ Zhiping Yang,¹ YaoYu Ning,¹ Guozhong Qin,¹ Raija Soininen,³ Karl Tryggvason,⁴ and Lester Kobzik¹,²

¹Physiology Program, Harvard School of Public Health and ²Department of Pathology, Brigham and Women’s Hospital, Boston, MA 02115
³Department of Medical Biochemistry and Molecular Biology, Biocenter Oulu, University of Oulu, 90014 Oulu, Finland
⁴Department of Medical Biochemistry and Biophysics, Karolinska Institute, 171 77 Stockholm, Sweden

Abstract

Alveolar macrophages (AMs) express the class A scavenger receptor macrophage receptor with collagenous structure (MARCO), but its role in vivo in lung defense against bacteria and environmental particles has not been studied. We used MARCO-deficient mice to directly test the in vivo role of AM MARCO in innate defense against pneumococcal infection and environmental particles. In a murine model of pneumococcal pneumonia, MARCO−/− mice displayed an impaired ability to clear bacteria from the lungs, increased pulmonary inflammation and cytokine release, and diminished survival. In vitro binding of Streptococcus pneumoniae and in vivo uptake of unopsonized particles by MARCO−/− AMs were dramatically impaired. MARCO−/− mice treated with the “inert” environmental particle TiO₂ showed enhanced inflammation and chemokine expression, indicating that MARCO-mediated clearance of inert particles by AMs prevents inflammatory responses otherwise initiated by other lung cells. Our findings point to an important role of MARCO in mounting an efficient and appropriately regulated innate immune response against inhaled particles and airborne pathogens.

Key words: macrophage • phagocytosis • environment • innate immunity

Introduction

Lung macrophages are both sentinels and the first line of defense against infection or injury from inhaled agents. The repertoire of receptors expressed on the alveolar macrophage (AM) surface is critical for binding, uptake, and response to microbes and inhaled unopsonized environmental particles. One family of receptors likely to be important for normal host defense of the lung are the class A macrophage scavenger receptors (SRas). This designation describes a group of pattern recognition receptors composed of three members as follows: SRA-I/II, macrophage receptor with collagenous structure (MARCO; reference 1), and the recently identified SRCL (scavenger receptor with C-type lectin; reference 2). All are multifunctional trimeric glyco-proteins expressed primarily by macrophages.

The class A scavenger receptors (especially the relatively well-studied SRA-I/II) have been implicated to varying degrees in systemic innate immunity against infection (3–10). Other data also suggest the potential involvement of MARCO in antibacterial defense mechanisms. For example, constitutive MARCO is up-regulated in liver and spleen macrophages after bacterial infection (11–13). Moreover, MARCO can bind LPS or LTA (14), as well as intact bacteria (Escherichia coli and Staphylococcus aureus; references 15, 16). MARCO is also expressed on dendritic cells (17) and may play a role in adaptive immunity. We have previously identified MARCO as a major receptor on AMs for in vitro binding of unopsonized environmental particles and certain microorganisms (15, 18), but its actual functional importance for lung host defense in vivo has not been investigated. Use of mice with genetic deletion of MARCO allowed specific analysis of its in vivo role in defense of the lung against two commonly encountered challenges, pneumococcal bacteria and inert environmental dusts.
Materials and Methods

**Animals.** 8-12-wk-old male mice genetically deficient in MARCO (MARCO⁻/⁻) were used in all experiments. Age- and sex-matched wild-type (C57BL/6 MARCO⁺/⁺) mice purchased from Charles River Laboratories were used as controls. MARCO⁻/⁻ mice were developed using targeted homologous recombination (unpublished data), and were backcrossed for at least eight generations to the C57BL/6 background. All animals were housed in sterile microisolator cages and had no evidence of spontaneous infection. Approval before all experimentation was obtained from the institutional animal use review committee.

**Reagents and Particles.** Titanium dioxide (TiO₂) particles (median diameter ~1 μm) were provided by J. Brain (Harvard School of Public Health, Boston, MA). Particles were suspended in BSS⁻ (124 mM NaCl, 5.8 mM KCl, 10 mM dextrose, and 20 mM Hepes) as stock solutions and sonicated (~30 s) before use. All reagents not otherwise specified were obtained from Sigma-Aldrich.

**Cell Isolation and Flow Cytometric Assay of Particulate Binding.** Mice were killed by i.p. pentobarbital injection. AMs obtained by repeated lung lavage with BSS⁻ were centrifuged at 150 g and resuspended in BSS⁺ Ca (0.3 mM) and Mg (1 mM). AMs (2 × 10⁵ in 100 μl BSS⁺) were preincubated with 2.5 μg/ml cytochalasin D for 5 min on ice in a 1-ml microfuge tube. After the addition of probe-sonicated particles, the tubes were rotated at 37°C for 30 min, placed on ice, and analyzed by flow cytometry. Flow cytometry was performed as described previously (15). AM uptake of particles was measured using the increase in the mean right angle scatter (RAS) caused by these granular materials (15).

**Particle Effects In Vivo.** Mice were lightly anesthetized with halothane, and solutions of particles in pyrogen-free saline were administered via intranasal insufflation (50 μl). Pilot experiments in normal wild-type mice showed minimal response to 5 mg/ml of the inert particle TiO₂. These concentrations were used in subsequent experiments comparing responses of MARCO-deficient mice. Mice were killed 4 h later. Bronchoalveolar lavage (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 BAL fluid were stored at ~70°C for the subsequent ELISA assay of TNF-α (R&D Systems). In some experiments, whole lung tissue was harvested without lavage for RNA extractions using the TRIzol reagent (GIBCO BRL). Expression of genes, whole lung tissue was harvested without lavage for RNA extractions using the TRIzol reagent (GIBCO BRL). Expression of probe-sonicated particles, the tubes were rotated at 37°C for 5 min, placed on ice, and analyzed by flow cytometry. Flow cytometry was performed as described previously (15). AM uptake of particles was measured using the increase in the mean right angle scatter (RAS) caused by these granular materials (15).

**Histopathology.** Parallel infection experiments were performed to harvest the lungs for histopathology. Age- and sex-matched MARCO⁻/⁻ and MARCO⁺/⁺ control mice were killed 24 h after inoculation. Lungs were harvested and kept in 10% formalin. Organs were examined histologically by hematoxylin and eosin staining. To quantitate AM number in situ, nonspecific esterase staining of cryostat sections of normal lung from wild-type or MARCO⁻/⁻ mice was performed. Random fields of alveolar parenchyma were analyzed (10 per sample, three mice per group). An index of esterase-positive macrophages (numerator) and alveolar tissue points (denominator) were calculated using a grid projection system, and the data were expressed as a ratio.

**Preparation of FITC-labeled Pneumococci.** S. pneumoniae were labeled with FITC (Molecular Probes). Bacteria were grown overnight on sheep blood agar plates, washed twice, and resuspended in PBS. Bacterial concentration was set to ~10⁸ CFU/ml as determined by OD₅₆₂. Bacteria were heat killed at 95°C for 10 min, centrifuged, and resuspended to 1 ml of labeling buffer (0.1 M NaHCO₃, pH 9.2). 0.25 mg of newly prepared FITC in DMSO was added per milliliter of suspension, 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 10⁸ bacteria/ml.

**Phagocytosis Assay.** Binding and phagocytosis of FITC-labeled pneumococci by AMs were quantified using a flow cytometric assay. BAL of MARCO⁻/⁻ and control mice was performed in PBS, and cells were washed and resuspended in BSS⁺. Aliquots of 0.5 ml (BAL cell count adjusted to 0.5 × 10⁶ cells/ml >95% macrophages, as judged by differential staining) were added to 1 ml conical microcentrifuge tubes together with FITC-labeled pneumococci at a bacteria/AM ratio of 50:1, incubated for 1 or 2 h at 37°C with continuous gentle rotation. Alternatively, AMs were preincubated with 10 μM cytochalasin D (Sigma-Aldrich) for 15 min at room temperature before the addi-
Results and Discussion

First, we sought to investigate whether the absence of MARCO receptor results in impaired bacterial clearance from the lungs of infected mice. S. pneumoniae, a relatively low inoculum likely to be cleared by normal AMs with minimal inflammation. Viable bacteria were quantitated in lung tissue homogenates obtained after 4 h. Wild-type MARCO+/+ mice showed efficient clearance of the bacteria, with only 8% of the inoculum being recovered on average (Fig. 1A). In contrast, >50% of the inoculated bacteria were still alive and recovered from the lungs of MARCO−/− mice (Fig. 1A). Similar results were seen at 24 h after intranasal administration (Fig. 1B).

BALF obtained 4 h after infection from MARCO−/− mice contained substantially more neutrophils compared with +/+ controls (Fig. 1, C and D, 17 ± 7.40 vs. 2.45 ± 0.46%, a finding corroborated by histologic analysis of infected lung tissue (Fig. 1, E and F). BAL fluid infected MARCO−/− mice also contained higher amounts of TNF-α and MIP-2 (0.58 ± 0.14 and 6.68 ± 0.94 pg/ml, respectively) than samples from control mice (Fig. 1 G, 0.13 ± 0.11 and 4.48 ± 0.39 pg/ml, respectively). A similar profile was observed 24 h after infection, though the overall levels of cytokines were lower (Fig. 1 H). Morphometric comparison of the tissue density of macrophages showed no significant differences between wild-type and MARCO−/− (1.027 ± 0.034 vs. 1.077 ± 0.028; mean ± SEM; P = 0.29 +/+ vs. −/−), a finding consistent with equal numbers of AMs recovered in BAL from normal MARCO++ and MARCO−/− mice (unpublished data). These data exclude simple deficiency in numbers of AMs as the mechanism for the differences observed in responses to bacteria (or particles) in the MARCO−/− mice.

A failure to eliminate S. pneumoniae reaching the bronchoalveolar space may lead to increased mortality from severe pneumonia or disseminated infection. To test this postulate, we investigated survival over time of MARCO+/+ and MARCO−/− mice inoculated with 2 × 10⁵ CFU of S. pneumoniae (a slightly higher dose required to detect...
mortality in the wild-type mice). Although mortality in the wild-type group was 40%, the total mortality in the MARCO−/− mice was 100% by day 10 (Fig. 2).

We postulate that these data correspond well to the natural history of pneumococcal infection in a nonimmune host. Pneumococcal infection is relatively common, but far less common than might be predicted from high rates of nasopharyngeal colonization in normal adults and children (20) and from the frequency of normal nocturnal aspiration of upper airway secretions (21). Resident AMs are the most important initial cellular defense against inhaled pathogens (22, 23). Our data indicate that MARCO is a critical receptor for this early defense mechanism. Once established, pneumococcal infection causes neutrophil influx in response to unchecked bacterial proliferation and tissue injury. In the absence of specific antibody, the PMN influx is relatively ineffective in restricting progression of the pneumonia as evinced in the high mortality of untreated pneumococcal pneumonia in people (24) and in the increased mortality in MARCO−/− mice seen in survival studies.

To more directly evaluate the contribution of MARCO receptor to AM binding and uptake of pneumococci, AMs from wild-type and MARCO−/− mice were incubated for 1 h with FITC-labeled S. pneumoniae in the absence or presence of cytochalasin D, and the samples were analyzed by flow cytometry. In the presence of cytochalasin D, only binding of fluorescent bacteria occurs because internalization is blocked (15, 25). Under these conditions, MARCO−/− AMs showed substantially diminished bacterial binding (Fig. 3, e.g., 80 vs. 162 MFI units). When both binding and internalization were intact (absence of cytochalasin D), AMs from wild-type mice exhibited almost a fourfold higher MFI value compared with the MARCO−/− mice AMs (Fig. 3, 323 vs. 90 MFI units). Calculation of the fluorescence attributable to internalization of bacteria by AMs shows a substantially lower amount in MARCO−/− mice (Fig. 2, inset, 161 vs. 10 MFI units). These data are consistent with the marked defect in clearance of bacteria and the resulting increased inflammatory response observed in vivo.

Nevertheless, the persistence of some residual binding indicates the presence of other receptors capable of binding to S. pneumoniae. Interestingly, the absence of MARCO also reduced internalization of the lower numbers of AM-bound S. pneumoniae. The basis for this indirect implication of MARCO in the internalization process for bound bacteria is unclear and warrants further study.

Next, we investigated in vivo responses to a prototypical "inert" particle (TiO₂), so named because usually it is rapidly cleared by AM phagocytosis with minimal or no pulmonary inflammation or injury (26, 27). BAL analysis 4 h later showed diminished uptake of particles by AMs from the MARCO−/− mice (Fig. 4 A). TiO₂ administration caused minimal PMN influx into BAL samples from wild-type control mice (Fig. 4 B). In contrast, MARCO−/−
mice showed a dramatic increase in PMN trafficking into the lungs (102.8 ± 39.8 × 10³ cells/ml), compared with the control mice (6.3 ± 1.2 × 10³ cells/ml) and to vehicle (PBS)-induced recruitment in both groups (Fig. 4 B). Analysis of BAL fluid from TiO₂-treated MARCO⁻/⁻ mice also showed a marked increase of TNF-α (837 ± 397 pg/ml), as compared with 100 ± 50 pg/ml in response to PBS (Fig. 4 C). In wild-type mice, the level of BALF TNF-α induced by TiO₂ (82 ± 85 pg/ml) was low and not different from the vehicle (PBS) control (84 ± 20 pg/ml). Analysis of cytokine mRNA expression using an RNase protection assay (Fig. 4 D) showed that TiO₂ challenge induced a higher rate of MIP-2 expression in the lung tissue from MARCO⁻/⁻ mice (relative increase after TiO₂ vs. PBS challenge, MARCO⁻/⁻ vs. MARCO⁺/⁺, 3.4 ± 1.4 vs. 0.6 ± 0.09).

To test the possibility that MARCO⁻/⁻ AMs show an altered cytokine response to TiO₂, we measured TNF-α and MIP-2 release by AMs treated in vitro with TiO₂. MARCO⁻/⁻ and +/+ AMs showed similar levels of increased cytokine release in response to the positive control, LPS, and similarly minimal release in response to TiO₂ (unpublished data). Also, AMs lavaged from MARCO⁻/⁻ mice 2 h after instillation of TiO₂ (before the large increase of PMNs seen at 4 h) also did not show any substantial increase in cytokine release after in vitro culture (unpublished data). These findings argue against an altered response in MARCO⁻/⁻ AMs as the source of mediators seen after in vivo TiO₂.

The enhanced inflammatory response seen with inert TiO₂ in MARCO⁻/⁻ mice is noteworthy. We postulate that the reduced binding of TiO₂ by MARCO⁻/⁻ AMs (as demonstrated in vivo; Fig. 4 A) allows increased interaction of TiO₂ with other lung cells (i.e., epithelium) due to delayed uptake by the AM, which in turn leads to the increased MIP-2 expression and neutrophil influx observed. In contrast with its lack of proinflammatory effects on AMs, TiO₂ has been shown to elicit release by lung epithelial cells of chemokines for neutrophils such as MIP-2 and other cytokines (28, 29).

These studies using MARCO-deficient mice show an important function for this AM class A scavenger receptor in host defense against pneumococcal pneumonia and against pulmonary inflammation from otherwise inert environmental dusts. Because SRA (including MARCO) expression levels can increase or decrease in response to various stimuli (30), the possibility that pathologic or therapeutic changes in MARCO expression might impact lung host defense merits further investigation.

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