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*J Immunol* 2010; 185:4328-4335; Prepublished online 1 September 2010; doi: 10.4049/jimmunol.1000989
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The Class A Scavenger Receptor, Macrophage Receptor with Collagenous Structure, Is the Major Phagocytic Receptor for Clostridium sordellii Expressed by Human Decidual Macrophages

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Clostridium sordellii is an emerging pathogen associated with highly lethal female reproductive tract infections following childbirth, abortion, or cervical instrumentation. Gaps in our understanding of the pathogenesis of C. sordellii infections present major challenges to the development of better preventive and therapeutic strategies against this problem. We sought to determine the mechanisms whereby uterine decidua macrophages phagocytose this bacterium and tested the hypothesis that human decidual macrophages use class A scavenger receptors to internalize unopsonized C. sordellii. In vitro phagocytosis assays with human decidual macrophages incubated with pharmacological inhibitors of class A scavenger receptors (fucoidan, polyinosinic acid, and dextran sulfate) revealed a role for these receptors in C. sordellii phagocytosis. Soluble macrophage receptor with collagenous structure (MARCO) receptor prevented C. sordellii internalization, suggesting that MARCO is an important class A scavenger receptor in decidual macrophage phagocytosis of this microbe. Peritoneal macrophages from MARCO-deficient mice, but not wild-type or scavenger receptor AI/II-deficient mice, showed impaired C. sordellii phagocytosis. MARCO-null mice were more susceptible to death from C. sordellii uterine infection than wild-type mice and exhibited impaired clearance of this bacterium from the infected uterus. Thus, MARCO is an important phagocytic receptor used by human and mouse macrophages to clear C. sordellii from the infected uterus. The Journal of Immunology, 2010, 185: 4328–4335.

The clostridia are anaerobic, spore-forming bacilli that cause a diverse array of toxin-mediated infections in humans, including botulism, tetanus, and antibiotic-associated diarrhea. * Clostridium sordellii is an emerging pathogen associated with highly lethal female reproductive tract infections, bacteremia, and soft tissue infections (1). The high mortality of C. sordellii infections is associated with a stereotypical toxic shock syndrome (2). A recent study estimated that nearly 1 in 200 deaths in women of reproductive age was associated with clostridial toxic shock, due to C. sordellii and/or the related Clostridium perfringens (3). An increased number of severe C. sordellii infections has been reported over the past decade, following childbirth and abortion (4–6). Gaps in our understanding of the pathogenesis of C. sordellii infections present major challenges to the development of better preventive and therapeutic strategies against this emerging problem.

Macrophages are important in defending the host against invasive clostridial infections (7), although the role of uterine macrophages in innate immune defense against clostridia is undefined. During pregnancy, uterine decidual macrophages (DMs) participate in immunosurveillance, binding, ingesting, and clearing bacteria that ascend beyond the cervix (8). The receptor-mediated mechanisms by which DMs recognize and phagocytose unopsonized bacteria, including clostridia, have not been closely analyzed.

The macrophage scavenger receptors are a large family of immunosurveillance receptors used to recognize and internalize unopsonized pathogens, apoptotic host cells, and modified lipoproteins (9, 10). They are increasingly recognized to play a critical role in the clearance of unopsonized bacterial and parasitic microorganisms. Scavenger receptors are transmembrane proteins belonging to at least eight different subclasses (A–H) based on their tertiary structure (9, 10). The class A scavenger receptors (CASRs) are widely expressed on macrophages (9) and have been shown to bind Gram-positive bacteria through the recognition of lipoteichoic acid (11, 12). For example, the CASRs have been implicated in the phagocytosis of Listeria monocytogenes (13), various streptococci (14, 15), and the clostridial pathogen C. perfringens (16). We therefore speculated that CASRs would be expressed by DMs and capable of internalizing C. sordellii.

Two particular CASR subtypes, scavenger receptor AI/II (SR-AI/II) and macrophage receptor with collagenous structure (MARCO), participate in macrophage phagocytosis of Gram-positive bacteria.

Received for publication March 26, 2010. Accepted for publication July 20, 2010.

This work was supported by National Institutes of Health Grants HD057176 (to D.M.A.), ES10088 (to L.K.), and HL056309 (to J.L.C.). Support for nonhuman portions of this work was provided by a Doris Duke Charitable Fund Clinical Scientist Development Award (to D.M.A.).

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Abbreviations used in this paper: AcLDL, acetylated low-density lipoprotein; CASR, class A scavenger receptor; CBSR, class B scavenger receptor; ChSO₄, chondroitin sulfate; DM, decidual macrophage; DαSO₄, dextran sulfate; KO, knockout; MARCO, macrophage receptor with collagenous structure; PI, phagocytic index; poly(C), polycytidylic acid; poly(I), polyinosinic acid; RFU, relative fluorescence unit; SCARA-5, scavenger receptor A5; sMARCO, soluble MARCO; SR-AI/II, scavenger receptor AI and AII; SRCL, scavenger receptor with C-type lectin; WT, wild type.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1000989
The SR-AI, II, and III isoforms are derived from the same gene (MSR1) on chromosome 8, with alternative splicing producing the different proteins (9). Of these splice variants, only SR-AI and II (referred to in this work as SR-AI/II) are phagocytic, whereas SR-AI/III is unable to bind extracellular ligands because it is retained within the endoplasmic reticulum (9). The SR-AI/II receptors have been implicated in the clearance of Gram-positive pathogens (14), and SR-AI was reported to bind to C. perfringens in transfected nonphagocytic cells (16).

A separate gene (MARCO) on chromosome 2 encodes the MARCO receptor (9). Emerging data also support a major role of the MARCO receptor in macrophage phagocytosis of Gram-positive bacteria. The transcription and cell surface expression levels of MARCO are enhanced upon bacterial binding and provide evidence for its significance in bacterial clearance (9). Studies of alveolar macrophages have identified MARCO to be a major receptor for unopsonized bacteria (15, 17).

In this light, we questioned whether human DMs use CASRs to bind and internalize unopsonized C. sordellii and further asked whether SR-AI/II and/or MARCO would be important receptor subtypes in this process. Alternatively, because class B scavenger receptors (CBSRs) and the macrophage mannose receptor have also been reported to be capable of phagocytosis of Gram-positive bacteria (16, 18), we questioned whether these receptors were also active in the clearance of C. sordellii.

To address these questions, we studied the expression of CASR and CBSR subtypes by human DMs and assessed their functional relevance in phagocytosis assays of C. sordellii in vitro. Macrophages derived from mice genetically lacking SR-AI/II or MARCO receptors were also used to examine the relevance of these receptors to immune clearance of C. sordellii. Lastly, a mouse model of C. sordellii uterine infection was used to define the importance of CASR subtypes in host defense. These studies shed new light onto the potential importance of CASRs in the innate immune defense against highly lethal clostridial infections of the female reproductive tract.

Materials and Methods

Animals

Six- to eight-week-old, female SR-AI/II−/−-deficient (SR-AI1−/−) mice on a 129/SvJ strain background were originally provided as a generous gift of W. de Villiers (University of Kentucky Medical Center, Lexington, KY). These were bred locally at our facility. Age- and sex-matched BALB/c WT mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Six- to eight-week-old, female mice genetically deficient in MARCO (MARCO−/−) on a BALB/c background were bred as previously published (19) and used with the generous permission of K. Tryggvason (Karolinska Institute, Stockholm, Sweden), who initially provided the MARCO−/− mice. The genotypes of mouse strains were confirmed by tail-snip DNA PCR analyses performed by Transnetx (Cordova, TN). Age- and sex-matched BALB/c WT mice were purchased from The Jackson Laboratory. Animals were treated according to National Institutes of Health guidelines for the use of experimental animals with the approval of the University of Michigan Committee for the Use and Care of Animals.

Bacteria

C. sordellii strain ATCC9714 was obtained from the American Type Culture Collection (Manassas, VA) and grown anaerobically in broth culture overnight at 37°C in reinforced clostridial medium. Estimates of bacterial concentrations were derived from the OD of bacterial cultures at 600 nm (OD600) and a previously determined standard curve of CFU versus OD600. Estimated bacterial concentrations were confirmed by serial 10-fold dilutions on solid agar composed of reinforced clostridial media containing 1.5% (w/w) agar, incubated anaerobically. For phagocytosis experiments (below), heat-killed C. sordellii were prepared by heating to 121°C for 30 min.

Reagents

RPMI 1640, penicillin/streptomycin/amphotericin B solution, TRIZol, and acetylated low-density lipoprotein (AcLDL) were from Life Technologies-Invitrogen (Carlsbad, CA). FBS and charcoal-stripped FBS were from HyClone Laboratories (Waltham, MA). Reinforced clostridial medium, mouse anti-human CD14 IgG (clone MR1999), and mAb CD11b (clone D12) were from BD Biosciences (San Jose, CA). Cytochalasin D, hyaluronidase, DNase, Percoll, fucoidan, polyniosinic acid [poly(I)], poly- cytidylic acid [poly(C)], dextran sulfate, chondroitin sulfate, mannan, FITC, and trypan blue were from Sigma-Aldrich (St. Louis, MO). Magnetic MACS CD14 microbeads (human) were from Miltenyi Biotec (Auburn, CA). Recombinant mouse soluble MARCO (sMARCO) and monoclonal anti-human CD163 (clone 215927) were purchased from R&D Systems (Minneapolis, MN). F4/80 (clone 6A545) monoclonal IgG was from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal CD36 IgG (clone FA6-152) was purchased from Abcam (Cambridge, MA).

Human subjects

Following appropriate informed consent, human decidual tissue was obtained from healthy adult women aged 18–44 y undergoing elective surgical termination of pregnancy during the first trimester. This study was reviewed and approved by the University of Michigan Institutional Review Board.

DM isolation

This procedure was adapted from a previously described protocol (20). Decidual tissue was collected from surgical abortions under aseptic conditions. The tissue was weighed and minced into small pieces, then subjected to a tissue digest with a solution containing 1 mg/ml collagenase from C. histolyticum type I-A (Sigma-Aldrich), 1 mg/ml hyaluronidase from bovine testes type I-S (Sigma-Aldrich), and 150 μg/ml DNase I from bovine pancreas type IV (Sigma-Aldrich). A total of 10 ml digestion solution was used per gram of tissue, and the samples were placed on a shaker at 37°C for 60 min. Samples were then washed using RPMI 1640 medium containing L-glutamine, which was substituted with 1% antibacterial-antimycotic (Life Technologies-Invitrogen) and centrifuged at 1500 rpm at 4°C for 10 min. This was followed with serial 200-, 200-, and 100-μm nylon mesh filtrations to eliminate any remaining particulates. The cells were washed, as previously described, and the filtrate was resuspended in 25% Percoll (Sigma-Aldrich) in RPMI 1640 (same as above) and overlaid onto 50% Percoll, with 2 ml PBS layered above the 25% Percoll. Decidual cells were recovered from the 25%/50% interface following a 4°C centrifugation for 45 min at 1500 rpm. These cells were washed as above, followed by a RBC lysis using NH4Cl/Tris-HCl. After two washes, the cells were then passed through a 30-μm falcon filter (BD Biosciences).

Using Abs for the typical macrophage markers CD14, F4/80, and CD11b, flow cytometry was employed to verify that macrophages make up ~10% of human decidual tissue (results not shown) (8). The isolated cells underwent a positive selection step for macrophages by passing through two successive large cell columns (Miltenyi Biotec) using MACS CD14 microbeads (Miltenyi Biotec). Flow cytometry was used to confirm the purity of the isolation, with >94% of cells CD14+ (results not shown).

Resident peritoneal macrophage isolation

Resident peritoneal macrophages from mice were obtained via peritoneal lavage, as previously described (21, 22). A RBC lysis was performed, and cells were resuspended in RPMI 1640 containing 10% FBS and 1% penicillin/streptomycin/amphotericin. Cells were plated in 384-well tissue culture-treated plates (Costar, Cambridge, MA) at 2 3 105 cells/well and incubated overnight (37°C with 5% CO2). The following day, cells were washed two times with warm medium to remove nonadherent cells.

RNA extraction, cDNA synthesis

TRIZol (Life Technologies-Invitrogen) was added to the CD14+ decidual cells to perform an RNA extraction. After complete dissociation, chloroform (Sigma-Aldrich) was added at 200 μl/ml TRIZol. Samples were centrifuged at 13,000 rpm for 15 min at 4°C, the aqueous phase was saved, and an equal volume of isopropanol was added. After 20-min incubation at 22°C, samples were centrifuged as before. Pellet was resuspended in 500 μl 80% ethanol and centrifuged as before. Diethylpyrocarbonate-treated water was added to pellet, and RNA concentration was determined by ratiometric light absorbance at 260/280 nm. Then 1 μg RNA was transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland), per the manufacturer’s instructions. Samples were run on a Mastercycler Eppendorf 5 (Life Technologies-Invitrogen) with the following conditions: 65°C for 10 min, 50°C for 60 min, and 85°C for 5 min.
Semiquantitative real-time PCR

Primers and probes were designed using the Roche Universal Probe Library Assay Design Center (www.roche-applied-science.com). All primers (Table I) were produced by Integrated DNA Technologies, and all probes were from Roche, Universal ProbeLibrary Reference Gene Assays (Roche) for human and mouse GAPDH were used, as appropriate. To prepare the assay, 2 µL cDNA aliquots were used to prepare assays, according to manufacturer’s instructions, and run on the LightCycler 480 (Roche) with the following conditions: 95°C, 10 min (preincubation); 95°C, 10 s; 60°C, 30 s; 72°C, 1 s (amplification, 45 cycles); 95°C, 10 s; 50°C, 30 s; 70°C, 5 min (melting curve); 40°C, 30 s (cooling). Analysis was performed using Roche software; all samples were referenced to the expression of the housekeeping gene GAPDH.

Phagocytosis assays

CD14+ cells were cultured overnight (37°C) in 384-well tissue culture-treated plates with 2 × 10^5 cells/well. The cells were treated with compounds of interest and incubated for 30 min at 37°C. Heat-inactivated C. sordellii were surfaced labeled with FITC per our previously published protocol (23). FITC-labeled C. sordellii (FITC C. sordellii) was applied at a multiplicity of infection of 300 bacteria:1 cell and incubated for 3 h at 37°C. Phagocytosis was quantified according to our published method of measuring intracellular fluorescence as a surrogate marker of bacterial ingestion by macrophages (24). The fluorescence of intracellular FITC C. sordellii was determined using a microplate fluorometer (485/535 nm, SPECTRAMax GEMINI EM; Molecular Devices, Sunnyvale, CA), according to our previously published method (24). Briefly, fluorescence data were expressed in arbitrary relative fluorescence units (RFU), which were then converted into a phagocytic index (PI). The PI represented the fluorescence of intracellular (phagocytosed) bacteria (RFUf) and was calculated by subtracting the fluorescence of extracellular bacteria (RFUex) from the total fluorescence of the well (RFUtot). The RFUf was estimated by treating some cells with the phagocytosis inhibitor, cytochalasin D (5 µg/mL; EMD Chemicals, Gibbstown, NJ), for 30 min prior to exposure to FITC C. sordellii (25). The RFUf was calculated from the cytochalasin-treated wells was subtracted from the RFUtot. Therefore, the PI = RFU = RFUf - RFUex - RFUex (24).

Select compounds tested included the nonspecific CASR-blocking agents fucoidan, poly(I), and dextran sulfate; the negative control compounds chondroitin sulfate and poly(C); the mannose receptor blocker mannan; fucoidan, poly(I), and dextran sulfate; the negative control compounds were surfaced labeled with FITC per our previously published protocol (23). Phagocytosis inhibitor, cytochalasin D (5 µg/mL; EMD Chemicals, Gibbstown, NJ), for 30 min prior to exposure to FITC C. sordellii (25). The RFUf was calculated from the cytochalasin-treated wells was subtracted from the RFUtot. Therefore, the PI = RFUf - RFUex - RFUex (24).

Intrauterine infection

C. sordellii was cultured anaerobically (24 h, 37°C) in 10 ml liquid-reinforced clostridial medium (Life Technologies-Invitrogen). The bacteria were then centrifuged (2000 rpm × 10 min), and the pellet was washed three times with sterile PBS and resuspended in 1 ml total volume of PBS. Dilutions were made in PBS to allow the inoculation of ~1 × 10^8 CFU directly into one horn of the mouse uterus, according to our previously published protocol (23), in a total volume of 35 µl. The actual inoculum was calculated by subtracting the fluorescence of extracellular bacteria (RFUex) from the total fluorescence of the well (RFUtot). The RFUex was estimated by treating some cells with the phagocytosis inhibitor, cytochalasin D (5 µg/mL; EMD Chemicals, Gibbstown, NJ), for 30 min prior to exposure to FITC C. sordellii (25). The RFUf was calculated from the cytochalasin-treated wells was subtracted from the RFUtot. Therefore, the PI = RFUf - RFUex - RFUex (24).

Intraperitoneal injection

C. sordellii was cultured anaerobically (24 h, 37°C) in 10 ml liquid-reinforced clostridial medium (Life Technologies-Invitrogen). The bacteria were then centrifuged (2000 rpm × 10 min), and the pellet was washed three times with sterile PBS and resuspended in 1 ml total volume of PBS. Dilutions were made in PBS to allow the inoculation of ~1 × 10^8 CFU directly into one horn of the mouse uterus, according to our previously published protocol (23), in a total volume of 35 µl. The actual inoculum was plated by serial 10-fold dilutions of the PBS suspension onto solid reinforced clostridial medium agar and counting individual colonies the next day. For survival experiments, the mortality from infection was monitored for 8 d following inoculation.

Bacterial clearance and dissemination studies

For studies of bacterial clearance and dissemination, mice were infected intraperitoneal as above with 1 × 10^5 CFU C. sordellii strain ATCC9714. The uterus and spleen were harvested 15, 24, or 48 h postinfection and mechanically homogenized in 1 ml sterile PBS. Then, 50 µl homogenized sample was cultured anaerobically on reinforced clostridial medium agar, and colonies were enumerated after 24 h. Results were expressed as CFU/ml homogenate.

Statistical analyses

Mean values were compared using a one-way ANOVA, followed by a Bonferroni correction or a Student t test, as indicated. Differences were considered significant if p ≤ 0.05. Comparison of survival curves for mice infected with C. sordellii was performed using a Mantel-Cox log-rank test. Experiments were performed on at least three separate occasions, unless otherwise specified. Unless otherwise noted, data are presented as mean values ± SEM.

Results

Fucoidan inhibits the phagocytosis of C. sordellii by human DMs

Because C. perfringens was reported to be phagocytosed predominantly through CASRs in nonreproductive tract macrophages (16), we hypothesized that human DMs used CASRs in phagocytosing C. sordellii. To test this, DMs were pretreated with fucoidan, an antagonist of CASRs that is not selective for the unique CASR subtype (26). A 30-min preincubation with fucoidan at 100 and 500 µg/mL dose dependently and significantly inhibited the phagocytosis of FITC C. sordellii by 75.8 ± 4.9% and 96.0 ± 2.0%, respectively (Fig. 1A). Pilot experiments demonstrated that maximal inhibition was obtained using 500 µg/mL (data not shown), so this concentration was used as a positive, comparative control in other phagocytosis assays.

Human DMs express CASRs

Having established a possible role for CASRs in immune recognition of C. sordellii, experiments were conducted to determine their expression levels in DMs using semiquantitative real-time PCR. Although a functional mannose receptor has been identified on human DMs (29), to our knowledge other phagocytic receptors, including the CASRs and CBSRs, have not been well characterized. Thus, mRNA was isolated from unstimulated human DMs, and real-time PCR was performed for the CASRs SR-A/I/I, MARCO, SR with C-type lectin (SRCL), and scavenger receptor A5 (SCARA-5) (Table I). SRCL is an endothelial CASR that has also been identified on alveolar macrophages (30), SCARA-5 was recently characterized in murine epithelial cells and was found to have similar binding properties to other CASRs (31). However, functional roles in microbial clearance for either SRCL or SCARA-5 receptors have not been identified for human phagocytes. The expression of two CBSRs implicated in binding bacteria, CD36 and CD163, was also evaluated (32, 33). These experiments revealed expression of multiple scavenger receptors (Fig. 1B). Of the CASRs, the expression of SR-A/I/I was greatest, being significantly higher than SCARA-5, which was only minimally expressed. SR-A/I/I transcripts were not significantly increased using a one-way ANOVA, followed by a Bonferroni correction or a Student t test, as indicated. Differences were considered significant if p ≤ 0.05. Comparison of survival curves for mice infected with C. sordellii was performed using a Mantel-Cox log-rank test. Experiments were performed on at least three separate occasions, unless otherwise specified. Unless otherwise noted, data are presented as mean values ± SEM.

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greater than mRNA levels of MARCO. We also found a significantly higher expression of the CBSR, CD163, when compared with CD36.

Multiple pharmacological antagonists of CASRs impair phagocytosis of C. sordellii by human DMs

The above data suggested that human DMs express functional CASRs, but these experiments were limited by the use of a single, nonselective CASR antagonist, fucoidan. Thus, phagocytosis assays were performed using additional, standard, nonselective CASR-blocking agents. When cells were preincubated with dextran sulfate (100 μg/ml), there was an 87.3 ± 6.1% decrease in phagocytic ability, whereas the negative control (but structurally similar) agent chondroitin sulfate (100 μg/ml) had no effect (Fig. 2A). Another classical CASR antagonist, poly(I), suppressed phagocytosis by 48.5 ± 6.2% compared with vehicle treatment, whereas the negative control compound poly(C) had no effect (Fig. 2B). The generalizability of these results for other types of macrophages was tested by conducting experiments using resident peritoneal macrophages isolated from female Wistar rats, which yielded similar results (data not shown). Whereas these observations further implicated CASRs in the phagocytosis of C. sordellii by human DMs, they did not differentiate among the various subtypes.

A previous study by O’Brien and Melville (16) identified a minor role for the mannose receptor in the macrophage phagocytosis of C. perfringens. However, we found no role for this receptor in the phagocytosis of C. sordellii when human DMs were preincubated (30 min) with the antagonist mannan (100 μg/ml; Fig. 2C). We also failed to identify a role for mannose receptors in C. sordellii phagocytosis using resident rat peritoneal macrophages (data not shown).

MARCO is the predominant phagocytic receptor for C. sordellii by human DMs

To determine which of the predominant CASRs expressed by human DMs (SR-AI/II and MARCO) is primarily responsible for internalizing C. sordellii, a previously published pharmacological approach to selectively antagonize these receptors was employed (34). Cells were preincubated (30 min) either with recombinant, mouse sMARCO or with the SR-AI/II–selective agent AcLDL (34). AcLDL had no effect on C. sordellii phagocytosis, whereas sMARCO inhibited phagocytosis by 79.5 ± 2.1% (Fig. 3A). These data implicated MARCO as the predominant phagocytic receptor for C. sordellii on human DMs.

Because DMs express CBSRs (Fig. 1B) and macrophage CBSRs have been shown to bind and phagocytose bacteria (18, 32, 33), we preincubated DMs with mAbs against human CD163 and CD36 (50 μg/ml) before measuring phagocytosis. Consistently, a small, but statistically significant inhibitory effect (~30% inhibition) on phagocytosis was observed when cells were treated with anti-CD36 preincubation DMs with mAbs against human CD163 and CD36 (50 μg/ml) before measuring phagocytosis. Consistently, a small, but statistically significant inhibitory effect (~30% inhibition) on phagocytosis was observed when cells were treated with anti-CD36 treatment, whereas the CD163 Ab had no effect (Fig. 3B). Thus, CD36 appears to play a minor role in phagocytosing C. sordellii.

MARCO, but not SR-AI/II receptors are critical for mouse macrophage phagocytosis of C. sordellii

The above experiments were limited by their pharmacological approach. The availability of CASR-deficient mice allowed an alternative approach to test the importance of these receptors in the phagocytosis of C. sordellii. Resident peritoneal macrophages obtained from SR-AI/II−/− mice, MARCO−/− mice, or respective WT animals were challenged with FITC C. sordellii, and their relative phagocytic capacity was determined. We observed that macrophages from SR-AI/II−/− mice exhibited ~3.18 ± 0.75-fold greater phagocytosis of C. sordellii compared with WT cells (p < 0.05; Fig. 4A). This phagocytic ability was inhibited >90% using sMARCO (data not shown). Real-time PCR was performed to determine whether there were compensatory changes in MARCO expression in the SR-AI/II−/− macrophages correlating with their enhanced phagocytic capacity. As shown (Fig. 4B), MARCO mRNA levels were, on average, 5.3-fold higher in SR-AI/II−/− macrophages versus WT cells (p < 0.05).

These data suggested that in the absence of the SR-AI/II receptors, MARCO was able to internalize C. sordellii. To test this, we compared phagocytosis of unopsonized FITC C. sordellii using WT and MARCO−/− peritoneal macrophages. As shown (Fig. 4A), phagocytosis was significantly lower in macrophages lacking...
Results are mean ± SEM of three to five independent experiments performed in octuple. B, mRNA expression of SR-AI/II or MARCO in mouse peritoneal macrophages was determined by semiquantitative real-time PCR, as detailed in Materials and Methods. Left panel, mRNA levels were compared between WT BALB/c peritoneal macrophages (black bars) and MARCO/−/− cells (gray bars). Right panel, mRNA levels were compared between WT 129/SvJ peritoneal macrophages (black bars) and SR-AI/II/−/− cells (striped bars). Data are normalized to GAPDH expression and represent the mean ± SEM of eight mouse samples per assay. *p < 0.05 versus WT cells by t test. KO, knockout cells.

FIGURE 4. MARCO is more important than SR-AI/II in the phagocytosis of C. sordelli. A, Left panel. Peritoneal macrophages from either WT BALB/c mice or MARCO/−/− mice were allowed to phagocytose FITC C. sordellii for 3 h. Right panel, Peritoneal macrophages from either WT 129/SvJ mice or SR-AI/II/−/− mice were allowed to phagocytose FITC C. sordellii for 3 h. The phagocytosis of FITC C. sordellii was quantified by fluorometry after 180 min, as detailed in Materials and Methods. B, mRNA levels were compared between WT BALB/c peritoneal macrophages (black bars) and MARCO/−/− cells (gray bars). Right panel, mRNA levels were compared between WT 129/SvJ peritoneal macrophages (black bars) and SR-AI/II/−/− cells (striped bars). Data are normalized to GAPDH expression and represent the mean ± SEM of eight mouse samples per assay. *p < 0.05 versus WT cells by t test. KO, knockout cells.

FIGURE 3. sMARCO and anti-CD36 IgG prevent the phagocytosis of C. sordellii by DMs. A, Human DMs were pretreated with sMARCO (100 μg/ml) or the SR-AI/II-binding agent AcLDL (100 μg/ml) for 30 min, and the phagocytosis of FITC C. sordellii was quantified by fluorometry after 180 min, as detailed in Materials and Methods. B, Human DMs were pretreated with mouse anti-human monoclonal IgG directed against CD36 or an isotype control at 50 μg/ml for 30 min, and the phagocytosis of FITC C. sordellii was quantified by fluorometry after 180 min, as detailed in Materials and Methods. Fucoidan pretreatment for 30 min at 500 μg/ml used as positive control for each. Data are representative from three independent experiments performed in octuplet with similar results. Results are expressed as a percentage of relative to untreated cells. ***p < 0.001 versus untreated cells.

Discussion

These studies newly define a potentially important role for MARCO receptors in the innate host defense against invasive C. sordellii infections of the female reproductive tract. Infections caused by toxigenic clostridia are emerging as important challenges to human health. Whereas the problem of clostridial infections is growing, there has been little research into the mechanisms detailing how the innate immune system recognizes and attempts to eliminate these potential pathogens. The increase in published reports of highly lethal C. sordellii gynecological infections in women of childbearing age (1, 3, 6) prompted further study of the pathogenesis of C. sordellii infections. To our knowledge, these studies show for the first time the receptors through which human macrophages internalize these toxigenic bacteria.

Macrophages are key sentinels of innate immunity that respond to microbial invaders by elaborating immunoregulatory mediators (cytokines, chemokines, and lipids), phagocytosing and killing potential pathogens, and presenting Ags to cells of the adaptive immune system. Recently, the TLRs 2 and 6 were found to participate in the immune response of macrophages to C. sordellii, through the activation of the transcription factor NF-κB (35). However, these studies did not address how macrophages phagocytose this bacterium.

Previous investigations with the related pathogen, C. perfringens, revealed that CASRs, and to a lesser extent the mannose receptor, were involved in the macrophage phagocytosis of this pathogen (16). Phagocytosis of C. perfringens was impaired in those studies using the nonspecific CASR-blocking agent fucoidan (16). However, that study did not establish the relative roles played by individual CASRs in C. perfringens uptake. Although the authors used Chinese hamster ovarian cells transfected with the mouse SR-AI receptor to demonstrate binding of C. perfringens to SR-AI, the capacity of SR-AI to mediate the internalization of this bacterium was not reported (16).

Based largely on the findings of these important studies of C. perfringens, we questioned whether CASRs would also be important for the phagocytosis of C. sordellii by human macrophages.
Similar to the approach of O’Brien and Melville (16), we incubated DMs with fucoidan and noted that this broad inhibitor of CASR phagocytosis was a potent blocker of *C. sordellii* internalization. We then used mRNA expression as a surrogate marker for phagocytic receptor expression by DMs. Using real-time PCR, we identified transcription of the *MSRI* and the MARCO genes, encoding SR-AI/II and MARCO, respectively. These studies newly documented the expression of SRCL on DMs, a receptor expressed primarily on nonhematopoietic cells (e.g., endothelial cells) (30, 36). We also investigated whether SCARA-5 was expressed by human DMs, as this receptor has not been well characterized in human cells and tissues (31). However, we did not observe significant expression of this receptor.

To confirm the results obtained with fucoidan, we used pairs of nonselective CASR-blocking agents and structurally matched, negative control compounds that do not bind to CASRs [e.g., poly(I)-poly(C) and dextran sulfate-chondroitin sulfate]. Indeed, phagocytosis was inhibited by both dextran sulfate and poly(I), but not by the negative control agent chondroitin sulfate or poly(C).

A study by Laskarin et al. (29) suggested the mannose receptor is a functional receptor on decidual mononuclear cells. The exact role of the mannose receptor in phagocytosis is uncertain with conflicting biochemical and genetic studies (37). Due to its decreased expression during inflammation (38), it has been suggested the mannose receptor may be more important for tissue homeostasis than for host defense (37). However, taking into consideration this information and the previously mentioned studies by O’Brien and Melville (16), this receptor was also tested to determine its importance in the binding and phagocytosis of *C. sordellii*. We found no major role for the mannose receptor in our experimental design.

Although controversial, data suggest that TLR2 ligation is involved in the ingestion of Gram-positive bacteria by phagocytic cells (39). It was also recently reported that TLR2 plays a role in bacterial phagocytosis by mouse trophoblast giant cells (40). Using blocking mAbs against TLR2 on human DMs and rat peritoneal macrophages, we have not observed an inhibitory effect on the phagocytosis of *C. sordellii* compared with isotype control IgGs (data not shown). These data suggest that TLR2 does not play a significant role in the internalization of *C. sordellii*. However, alternative approaches, such as the use of macrophages from TLR2-deficient mice, or the use of genetic silencing in human cells, could provide additional evidence in this regard.

Given the potentially critical role for CASRs in the macrophage phagocytosis of *C. sordellii*, we sought to decipher which specific subtypes were most important. There is precedent to suggest that different pathogens may bind unique CASRs with varying efficiency (41, 34). We initially incubated human DMs with either AcLDL, a specific antagonist of SR-AI/II receptors (34), or sMARCO, a specific inhibitor of MARCO-dependent phagocytosis (34, 42). These studies found that phagocytosis could be potently blocked by sMARCO, but not by AcLDL, suggesting that MARCO is the more critical receptor for the phagocytosis of *C. sordellii*.

These pharmacological results were confirmed using receptor-deficient mice. We were surprised to observe a significantly greater phagocytic capacity in SR-AI/II−/− macrophages compared with WT cells (Fig. 4) and speculated that this might be due to enhanced MARCO expression, which has been observed in SR-AI/II−/− mice, although on a different genetic background (43). The fact that sMARCO inhibited the phagocytic capacity of the SR-AI/II−/− macrophages suggested this might be the case (data not shown). Indeed, mRNA transcript levels for MARCO were significantly greater in SR-AI/II−/− macrophages compared with WT macrophages (Fig. 4), providing more evidence that MARCO expression is upregulated in mouse cells lacking SR-AI/II. These data were limited by the fact that mRNA transscripts were quantified, not surface-expressed protein levels. However, a previous study of SR-AI/II−/− peritoneal macrophages (on a BALB/c background) showed higher surface expression of MARCO using an anti-MARCO Ab (43).

Notably, MARCO−/− peritoneal macrophages demonstrated an impaired capacity to phagocytose *C. sordellii*. These data supported our pharmacological results in the human cell. What is more, we observed, for the first time, a functional role for MARCO in immune defense against clostridia in vivo. Importantly, mice lacking functional MARCO receptors demonstrated increased susceptibility to lethal infection by *C. sordellii* and impaired clearance of these bacteria from the uterus (Fig. 5A, 5B). There was also a nonstatistically significant trend toward greater dissemination to the spleen in the MARCO−/− mice. As expected, we did not see enhanced susceptibility to infection in SR-AI/II null mice (Fig. 5C).

Whereas our mouse uterine infection model data provide novel and correlative support for our in vitro results, suggesting that MARCO is an important component of the innate defense against *C. sordellii* infections, they do not prove that DMs per se are involved in host defense in vivo. The complex cellular defense mechanisms involved in innate immunity against *C. sordellii* remain to be fully defined. We previously reported that human β defensin antimicrobial peptides were rapidly bactericidal against *C. sordellii* (23), suggesting that epithelial cells might be important in controlling *C. sordellii* infection within the uterus. Although not presented in this work, we have examined whether *C. sordellii* can invade (or get phagocytosed by) uterine epithelial cells. Histological examination of mouse uterine tissues 48 h.
following intrauterine inoculation with C. sordellii did not reveal bacteria within uterine epithelial cells in situ, and we have not observed significant phagocytosis of C. sordellii by the human uterine epithelial RL95-2 cell line in vitro (data not shown). What is more, using real-time, RT-PCR, we have found that RL95-2 cells do not express MARCO or SR-AI/II, although they do express SRCL, suggesting that SRCL is not an efficient phagocytic receptor for C. sordellii on these cells (data not shown).

Our data are in accord with previously published studies of the role of MARCO in immune defense against bacterial pathogens of the lung (44). In a murine model of pneumonia caused by the Gram-positive pathogen *Streptococcus pneumoniae*, MARCO-deficient mice displayed an impaired ability to clear bacteria from the lungs and significantly diminished survival (44). Our findings imply that genetic differences in MARCO expression or function might influence the risk of *C. sordellii* or other clostridial infections.

There are several limitations in our work. The bacteria used in our studies of phagocytosis were heat-killed, FITC-labeled *C. sordellii*, which would not be found in nature. Thus, these results may be different using live bacteria. In addition, the bacteria used were in a vegetative (nonsporulating) form, which models active invasion with replicating bacteria. However, future studies of the phagocytosis of spore forms will be of interest as well. Our study was also limited by the fact that we did not differentiate between the mRNA expression of SR-AI and SR-AII. These receptors are transcribed from the same gene and differ as a result of alternative mRNA splicing (45). An additional caveat is that the rodent cells used for these studies were peritoneal macrophages, which may differ significantly from reproductive tract cells. Thus, direct comparisons with human DMs are difficult.

These studies newly described expression of SRCL by human DMs. This receptor, to our knowledge, has not been shown to be a functional phagocytic receptor on macrophages. We did not examine its role in the phagocytosis of *C. sordellii*, and this will be a subject of future research. We also did not identify a major role for CBSRs in the phagocytosis of *C. sordellii* using mAbs to block CD36 and CD163. This approach has limitations, and future studies using genetically deficient, mouse-derived macrophages, or gene-silencing approaches would be necessary to confirm our results. It is notable that human DMs express relatively high levels of mRNA for the CBSR CD163 [transcripts for CD163 were the highest of all scavenger receptors that we examined by semiquantitative real-time PCR (Fig. 1B)]. The CD163 receptor is involved primarily in the clearance of hemoglobin–haptoglobin complexes (46). It has been implicated in anti-inflammatory responses and is a surface marker of alternative (M2) macrophage activation (46). Thus, our finding of CD163 expression by human DMs provides further support to other studies demonstrating that DMs exhibit features of alternative (M2) activation (47, 48). The implications of this for bacterial infections in the postpartum or postabortion period are unclear.

Studies of how DMs interact with pathogenic bacteria contribute to our understanding of these complex phagocytes. Mounting evidence suggests that DMs are important in host defense against bacterial infections during pregnancy, such as the Gram-positive pathogen *Listeria monocytogenes* (49). Whereas the clearance of bacteria may prevent overt intrauterine infection, the microbial activation of DMs can also drive preterm birth by stimulating the production of cytokines and PGs that cause uterine contraction and cervical dilation (50). In addition, DMs suppress immune responses at the fetal/maternal interface to induce and maintain maternal tolerance to the developing fetus (51, 52). Thus, unlike other macrophages, DMs defend against microbial invasion while maintaining an immunosuppressive phenotype in the context of maternal-fetal development. Future studies are needed to determine whether the immunosuppressive (M2) phenotype of DMs contributes to the enhanced susceptibility of pregnant women to intrauterine infection.

In summary, we newly characterize scavenger receptors expressed by human DMs and their relative importance in the recognition and internalization of the toxigenic pathogen *C. sordellii*. Using a combination of in vitro and in vivo experiments, we demonstrate a role for MARCO in reproductive tract innate immunity. These results may prove to be important not only for understanding the pathogenesis of invasive clostridial infections, but also for developing better preventive and therapeutic measures against a range of both sexually and nonsexually transmitted infections.

**Disclosures**

The authors have no financial conflicts of interest.

**References**

23. Aronoff, D. M., Y. Hao, J. Chung, N. Coleman, C. Lewis, C. M. Peres,

34. Plüddemann, A., S. Mukhopadhyay, M. Sankala, S. Savino, M. Pizza, R. Rappuoli,
33. Fabriek, B. O., R. van Bruggen, D. M. Deng, A. J. Ligtenberg, K. Nazmi,
32. Jiang, Y., P. Oliver, K. E. Davies, and N. Platt. 2006. Identification and char-
31. Jiang, Y., P. Oliver, K. E. Davies, and N. Platt. 2006. Identification and char-


32. Jiang, Y., P. Oliver, K. E. Davies, and N. Platt. 2006. Identification and char-
31. Jiang, Y., P. Oliver, K. E. Davies, and N. Platt. 2006. Identification and char-


32. Jiang, Y., P. Oliver, K. E. Davies, and N. Platt. 2006. Identification and char-
31. Jiang, Y., P. Oliver, K. E. Davies, and N. Platt. 2006. Identification and char-


32. Jiang, Y., P. Oliver, K. E. Davies, and N. Platt. 2006. Identification and char-
31. Jiang, Y., P. Oliver, K. E. Davies, and N. Platt. 2006. Identification and char-


32. Jiang, Y., P. Oliver, K. E. Davies, and N. Platt. 2006. Identification and char-
31. Jiang, Y., P. Oliver, K. E. Davies, and N. Platt. 2006. Identification and char-


32. Jiang, Y., P. Oliver, K. E. Davies, and N. Platt. 2006. Identification and char-
31. Jiang, Y., P. Oliver, K. E. Davies, and N. Platt. 2006. Identification and char-
