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Receptor-dependent phagocytosis of *Clostridium sordellii* by human decidual macrophages

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Receptor-Dependent Phagocytosis of *Clostridium sordellii* by Human Decidual Macrophages

by

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Abbreviations: AcLDL, acetylated low density lipoprotein; CASR, class A scavenger receptor; CBSR, class B scavenger receptor; DM, decidual macrophage; FITC, fluoroscein isothiocyanate; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; KO, knockout; poly(I), polyinosinic acid; poly(C), polycytidylic acid; SR-AI/II, scavenger receptor AI and AII; MARCO, macrophage receptor with collagenous structure; SRCL, scavenger receptor with C-type lectin; SCARA-5, scavenger receptor A5; TLR, toll-like receptor; WT, wild type.

Abstract

Clostridium sordellii is an emerging pathogen associated with highly-lethal female reproductive tract (FRT) 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 DMs phagocytose this bacterium and tested the hypothesis that human DMs utilize class A scavenger receptors (CASRs) to internalize unopsonized *C. sordellii*. *In vitro* phagocytosis assays with human DMs incubated with pharmacological inhibitors of CASRs (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 CASR in DM phagocytosis of this microbe. Peritoneal macrophages from MARCO-deficient mice, but not wild-type or SR-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 utilized by human and mouse macrophages to clear *C. sordellii* from the infected uterus.

Table of Contents

List of Figures

Chapter 1: Introduction

A recent increase in *Clostridium sordellii* uterine infections in pregnant women following medical abortion, spontaneous abortion, or natural childbirth has sparked interest into the question of how these infections occurred. Although *C. sordellii* infections in the FRT are rare, they are highly and rapidly lethal. Little is known about the pathogenesis of *C. sordellii* uterine infections, and even less is known about how innate immune cells of the uterus, such as DMs, interact with this anaerobic pathogen.

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. *C. sordellii* is an emerging pathogen associated with highly-lethal FRT infections, bacteremia, and soft tissue infections (2). The high mortality of *C. sordellii* infections is associated with a stereotypical toxic shock syndrome (5). A recent study estimated that nearly 1 in 200 deaths in women of reproductive age were associated with clostridial toxic shock, due to *C. sordellii* and/or the related *C. perfringens* (25). An increased number of severe *C. sordellii* infections has been reported over the past decade, following childbirth and abortion (21, 24, 43). 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.

In this light, **we hypothesized that human DMs utilize CASRs to bind and internalize unopsonized *C. sordellii***. We 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 (35, 45), we questioned whether these receptors were also active in the clearance of *C. sordellii*.

To address our major hypothesis and these related questions, we studied the expression of class A and B SR 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*. Last, a mouse model of *C. sordellii* uterine infection was utilized 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 FRT.

Chapter 2: Background

Clostridium sordellii

The anaerobic, gram-positive bacterium *Clostridium sordellii*, though rarely a human pathogen, is increasingly recognized as a cause of acute, fulminant, and highly lethal infections. Progress in developing preventive and therapeutic strategies against this pathogen has been hampered by a lack of research into its ecology and pathogenesis.

C. sordellii is rod-shaped and endospore-forming (40) and is normally found in the soil, within the intestinal flora of domestic animals, and has been estimated to colonize the gastrointestinal tract of 0.5% of humans (2). In animals (particularly cattle), a *C. sordellii* infection can cause fatal myositis, liver disease, enteritis, and sudden death. In humans it has been reported to cause pneumonia, endocarditis, arthritis, endometritis, peritonitis, myonecrosis, sepsis, toxic shock, and sudden death (3, 40). These infections have complicated various types of trauma, surgery, childbirth, spontaneous and medical abortion, and injection drug use, among others (2). Although in some cases the method of infection is known (*e.g.* the contamination of intravenously injected heroin or the transplantation of contaminated allograft materials), there are clinical scenarios for which the exact mechanism of infection remains speculative (*e.g.* following childbirth).

The virulence of *C. sordellii* is attributed to the many toxins it is able to produce, including: lethal toxin, hemorrhagic toxin, hemolysin, neuraminidase, phospholipase C, DNAse, hyaluronidase, and collagenase. Of these eight toxins, the most significant and well studied is undoubtedly lethal toxin.

Human Decidual Macrophages

Macrophages are important in defending the host against invasive clostridial infections (34), although the role of uterine macrophages in innate immune defense against clostridia is undefined. During pregnancy, uterine DMs participate in immunosurveillance, binding, ingesting, and clearing bacteria that ascend beyond the cervix (44). The receptor-mediated mechanisms by which DMs recognize and phagocytose unopsonized bacteria, including clostridia, have not been closely analyzed.

Clinical Significance

In recent years, increases in cases of toxic shock syndrome due to *C. sordellii* infections following childbirth and abortion have been reported. These cases were usually accompanied by signs such as tachycardia, hypotension, edema, hemoconcentration, profound leukocytosis, and absence of fever (21). It is thought that pregnancy, childbirth, and abortion each predispose women to *C. sordellii* infections by allowing the ascending infection of necrotic decidual tissue upon dilatation of the cervix (21). Interestingly, the majority of cases associated with medical abortion (7 of 8 reported cases) involved the intravaginal application of misoprostol, the synthetic prostaglandin (PG)E_2 analogue that is used in conjunction with the orally administered anti-progesterone drug mifepristone (RU-486) (2, 21), suggesting that one additional risk factor for *C. sordellii* uterine infection might be the suppression of FRT innate immunity by high local concentrations of a PGE_2-like compound (7). However, this has not been definitively established.

Macrophage Receptors Involved in Phagocytosis

The process of phagocytosis consists of three main steps: binding of a ligand (*e.g.* pathogen), cell activation and membrane motility, and entry into the cytoplasm (19). The initial means of binding is done through receptors located on the cell surface. These receptors are generally divided into the two categories of opsonic (*e.g.* FcγR, complement receptors, and CD14) or non-opsonic (*e.g.* mannose, scavenger, and complement receptor 3, dectin 1) (19).

One class of non-opsonic phagocytic receptors that is of particular importance is the SR. The SRs are transmembrane proteins that can be divided into at least eight different subclasses (A-H) based on their tertiary structure (41, 42). The CASRs are of special interest because they are widely expressed on macrophages (32). These receptors have previously been shown to bind Gram-positive bacteria through the recognition of lipoteichoic acid (18, 22) and may therefore play a key role in the phagocytosis of *C. sordellii*. In 2003, Melville and O'Brien demonstrated the importance of the CASR, and to a lesser degree the mannose receptor, in the binding and phagocytosis of *C. perfringens* (35). With the use of the CASR inhibitor, fucoidan, they found an 80% reduction in bacterial cell association. Reductions of 50% and 20% were also observed with the mannose inhibitors methyl-α-D-mannopyranoside and mannan, respectively (35).

The CASRs are subdivided even further into five different groups: SR-AI, SR-AII, SR-AIII, macrophage receptor with collagenous structure (MARCO), and scavenger receptor with C-type lectin (SRCL) (32). The SR-AI, II, and III isoforms are derived from the same gene on chromosome 8 with alternative splicing producing the different proteins (32). A separate gene encodes the MARCO receptor and is located on chromosome 2 (32). SRCL is

an endothelial CASR that has also been identified on alveolar macrophages (42). An additional CASR , SCARA5, recently characterized in murine epithelial cells associated with mucosal surfaces, was found to have similar binding properties as other CASRs (27). Functional roles in microbial clearance for either SRCL or SCARA5 receptors have not been identified for human phagocytes.

Extensive data support a functional role of the MARCO receptor in macrophage phagocytosis outside of the FRT. The cell surface expression and mRNA levels of MARCO are enhanced upon bacterial binding and provide evidence for its significance in bacterial clearance (32). In addition, studies using alveolar macrophages found MARCO to be the major receptor for unopsonized bacteria (10, 36). Thus, it is probable that human DMs are using the CASRs, possibly including MARCO receptors, to bind and engulf *C. sordellii*. However, a lack of information exists showing the presence or absence of CASRs on DMs.

A study by Laskarin *et al.* suggested the mannose receptor is a functional receptor on decidual mononuclear cells (29). The exact role of the mannose receptor in phagocytosis is uncertain with conflicting biochemical and genetic studies (19). Due to its decreased expression during inflammation (16), it has been suggested the mannose receptor may be more important for tissue homeostasis than for host defense (19). However, taking into consideration this information and the previously mentioned studies by O'Brien and Melville, this receptor was also tested to determine its importance in the binding and phagocytosis of *C. sordellii*.

Broader Relevance to Human Health

Although *C. sordellii* infections are uncommon, other bacterial infections that occur more commonly within the FRT cause an array of frequently encountered complications. These complications occur primarily due to the inability of our innate immune system to fend off the infection. This can result in many afflictions, including ectopic pregnancy, miscarriage, stillbirth, and congenital infections, which can result in chronic disability (*e.g.* infertility) and death. When a bacterial infection reaches the upper FRT, it can cause pelvic inflammatory disease. Twenty percent of women who develop pelvic inflammatory disease become infertile (50). These bacterial infections can also cause endometritis, chorioamnionitis, and pre-term births, resulting in approximately 70% of perinatal deaths (49). Determining the mechanism behind *C. sordellii* infections and their relationship with prostaglandins will expand not only our knowledge in bacterial pathogenesis, but also our understanding of the immunology of the FRT in general.

Chapter 3: Materials and Methods

Animals

Six 8-week-old, female SR-AI/II/II deficient (SR-AI/II$^{-/-}$) mice on a 129/SvJ strain background were originally provided as a generous gift of Dr. Willem J. S. de Villiers (University of Kentucky Medical Center, Lexington, KY, USA) and were subsequently bred at the University of Michigan. Age- and sex-matched 129/SvJ wild-type (WT) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Six 8-wk-old, female mice genetically deficient in MARCO (MARCO$^{-/-}$) on a BALB/c background were bred as previously published (9) and utilized with the generous permission of Dr. Karl 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 Transnetyx (Cordova, TN). Age- and sex-matched BALB/c WT mice were purchased from 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^{o}C in reinforced clostridial medium (RCM) (BD Biosciences). Estimates of bacterial concentrations were derived from the optical density (OD) of bacterial cultures at 600 nm (OD_{600}) and a previously-determined standard curve of colony forming units (CFU) *vs.* OD_{600}. Estimated bacterial concentrations were confirmed by serial 10-fold dilutions on solid

agar composed of RCM 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). Fetal bovine serum (FBS) and charcoal-stripped FBS were from HyClone Laboratories (Waltham, MA). RCM, mouse anti-human CD14 IgG (clone MPHIP9), and monoclonal antibody CD11b (clone D12) were from BD Biosciences (San Jose, California). Cytochalasin D, hyaluronidase, deoxyribonuclease, Percoll®, fucoidan, polyinosinic acid (Poly(I)), polycytidylic acid (poly(C)), dextran sulfate, chondroitin sulfate, mannan, fluorescein isothiocyanate (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, California). 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 years undergoing elective surgical termination of pregnancy

during the first trimester. This study was reviewed and approved by the University of Michigan Institutional Review Board.

Decidual Macrophage Isolation

This procedure was adapted from a previously described protocol (47). First trimester decidual tissue was collected from surgical abortions under sterile conditions. The tissue was weighed, minced into small pieces, and the tissue digested with a solution containing 1 mg/ml collagenase (Sigma), 1 mg/ml hyaluronidase (Sigma), and 150 μg/ml DNase (Sigma). Ten mls of digestion solution were used per gram of tissue. The samples were digested on a shaker at 37°C for 60 minutes. The sample was washed using RPMI medium 1640 containing L-glutamine (Gibco) and 1% antibiotic-antimycotic (Gibco) and centrifuged at 365 x g RCF at 4°C for 10 min. This was followed with a series of 280 μm, 200 μm, and 100 μm nylon mesh filtrations to eliminate any remaining particulates. The cells were washed as previously described and the filtrate was suspended in 25% Percoll (Sigma) in RPMI (same as above) and overlaid onto 50% Percoll, with 2 mls PBS layered above the 25% Percoll. Decidual cells are recovered from the 25/50 interface following a 4°C centrifugation for 45 minutes at 365 x g RCF. These cells were washed as above, followed by a red blood cell lysis using NH_4Cl/Tris-HCl. After two washes with RPMI medium 1640 containing L-glutamine (Gibco) and 1% antibiotic-antimycotic (Gibco), the cells were then passed through a 30 μm falcon filter (BD Biosciences) to remove any cellular clumps and/or debris.

Using typical macrophage markers, including $CD14^+$ (Becton Dickinson), $F4/80^+$ (Santa Cruz), and $CD11b^+$ (Becton Dickinson), we were able to verify that macrophages make up ~10% of human decidual tissue (results not shown). These results are in accordance

with previous findings (44). The isolated cells underwent a positive selection 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 our 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 (12, 37). A red blood cell lysis was performed and cells were suspended in RPMI 1640 containing 10% FBS and 1% penicillin/streptomycin/amphotericin. Cells were plated in 384-well tissue culture-treated plates (Costar) at 2 x 10^5 cells/well and incubated overnight (37°C with 5% CO_2). The following day cells were washed two times with warm RPMI to remove nonadherent cells.

RNA Extraction, cDNA Synthesis

TRIzol® (Invitrogen) was added to the CD14+ cells to perform an RNA extraction. After complete dissociation, chloroform (Sigma) was added to a final concentration of 200 µl/ml Trizol®. Samples were centrifuged at 16,000 x g RCF for 15 min at 4°C, the aqueous phase was taken off, and an equal volume of isopropanol was added. After 20 minutes incubation at room temperature, samples were centrifuged at 16,000 x g RCF for 15 min at 4°C. The pellet was suspended in 500 µl 80% ethanol and centrifuged at 16,000 x g RCF for 15 min at 4°C. DEPC water was added to the pellet, and RNA concentration was determined via nanodrop. 1 µg RNA was transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche) per manufacturer's instructions. Samples were run on a

Mastercycler epgradient S (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 Universal ProbeLibrary Assay Design Center at www.roche-applied-science.com. All primers were from Integrated DNA Technologies (Coralville, IA), and all probes were from Roche (Table 1). Universal ProbeLibrary Reference Gene Assays for GAPDH were used for human and mouse (Roche). To prepare the assay, 2 µl cDNA were used according to manufacturer's instructions and run on the LightCycler 480 (Roche) with the following conditions: 95°C 10 min (preincubation), 95°C 10 sec, 60°C 30 sec, 72°C 1 sec (amplification, 45 cycles), 95°C 10 sec, 50°C 30 sec, 70°C 5 min (melting curve), 40°C 30 sec (cooling). Analysis was performed using Roche software; all samples were referenced to the expression of the housekeeping gene *GAPDH*.

Table 1: Primers and Probes used for PCR amplification of SRs.

Gene	**Forward Primer**	**Reverse Primer**	**UPL Probe #**
MSR1	tcccactggagaaagtggtc	ctccccgatcacctttaagac	85
MARCO	tgctgggttactccaaagga	cagccagatctgcccagt	24
SCARA5	tgggcatcttcatcttagca	cattcagccggttcacatt	24
CD36	tggaacagaggctgacaactt	ttgattttgatagatatgggatgc	76
CD163	tggggaaagcataactgtga	gctcagatctgctccctttg	67
COLEC12	cagagcgtgaaaatgaatgg	tgtccagctttccaatttttg	42
Msr1 (*M. musculus)*	ctttaccagcaatgacaaaagaga	atttcacggattctgaactgc	89
Marco (*M. musculus)*	ggcaccaagggagacaaa	tcccttcatgcccatgtc	97

Phagocytosis Assays

$CD14^+$ cells were cultured overnight in 384-well tissue culture-treated plates (Costar) with 2 x 10^5 cells/well at 37°C. The cells were treated with specific compounds of interest and incubated for 30 min at 37°C. Heat-inactivated *C. sordellii* were surfaced-labeled with FITC per previously published protocol (11). FITC-labeled *C. sordellii* (^{FITC}*C. sordellii*) were applied at a multiplicity of infection (MOI) of 300 bacteria:1 cell and incubated for 3 hrs at 37°C. Trypan blue (250 μg/ml; Molecular Probes) was added to quench extracellular bacteria including those bound to the cell that had not been internalized. Phagocytosis was quantified according to a previously published method (7) by determining the fluorescence of intracellular bacteria within DMs using a microplate fluorometer ($485_{ex}/535_{em}$, SPECTRAMax GEMINI EM; Molecular Devices, Sunnyvale, CA). The data received are

expressed in arbitrary relative fluorescence units (RFU), which were converted into a phagocytic index (PI). The PI represents only bacteria that were intracellular (RFU_i) and is calculated by subtracting the extracellular bacteria (RFU_{ex}) from the total fluorescence of the well (RFU_{total}). The RFU_{ex} was determined by treating 3-4 wells with a phagocytic inhibitor, cytochalasin D (5 µl/ml; EMD chemicals), for 30 min prior to exposure with *C. sordellii*. The fluorescence from these wells represents unquenched extracellular bacteria and was subtracted from the $RFU_{total.}$ Therefore, the PI = RFU_i = RFU_{total} - RFU_{ex} (6). Control wells containing only cells and media were used to determine the amount of background fluorescence; this RFU value was subtracted from all other values to compensate for this fluorescence.

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; sMARCO; and the SR-AI/II blocking agent AcLDL. CBSR-blocking antibodies used included the mouse monoclonal anti-human CD163 and CD36 IgGs (33). Phagocytosis assays with mouse macrophages were similarly performed; however, a MOI of 150:1 was used. In addition, for assays comparing wild-type DM cells with either $MARCO^{-/-}$ or $SR\text{-}AI/II^{-/-}$, the results were normalized to total intracellular lactate dehydrogenase activity levels in control wells without bacteria to account for potential differences in cell number or adherence to tissue culture plates (13). Lactate dehydrogenase activity was determined by a commercially available assay according to the manufacturer's instructions (Roche).

Intrauterine Infections

C. sordellii was cultured anaerobically (24 hr, 37°C) in 10 ml RCM broth (BD Biosciences). The bacteria were then centrifuged (650 x g RCF x 10 min) and the pellet washed three times with sterile phosphate buffered saline (PBS) and suspended in 1 ml total volume of PBS. Dilutions were made in PBS to allow the inoculation of ~1 x 10^4 CFU directly into one horn of the mouse uterus, according to our previously published protocol (7), in a total volume of 35 μl. The actual inoculum was determined by plating serial 10-fold dilutions of the PBS suspension onto RCM agar and counting individual colonies the next day. For survival experiments, the mortality from infection was monitored for eight days following inoculation.

Bacterial Clearance and Dissemination Studies

For studies of bacterial clearance and dissemination, mice were infected intrauterine as above with 1 x 10^3-10^4 CFU *C. sordellii*. The uterus and spleen were harvested 24 hrs after infection and mechanically homogenized in 1 ml of sterile PBS. Then, 50 μl of homogenized sample was cultured anaerobically on RCM agar, and colonies were enumerated after 24 hrs. Results were expressed as CFU per ml of homogenate.

Statistical Analyses

Mean values were compared using a one-way analysis of variance (ANOVA) followed by a Bonferroni correction or a paired Student *t*-test as indicated. Differences were considered significant if $P \leq 0.05$. 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 ± standard error of the mean (SEM).

Chapter 4: Results

Fucoidan inhibits the phagocytosis of C. sordellii by human DMs

Because *C. perfringens* was reported to be phagocytosed predominantly through CASRs in non-reproductive tract macrophages (35), we hypothesized that human DMs utilized 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 subtypes (15). 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 $\pm$ 4.9% and 96.0 $\pm$ 2.0%, respectively (**Fig. 1A**). Pilot experiments demonstrated that maximal inhibition was obtained using 500 μg/ml of fucoidan (data not shown), so this concentration was used as a positive, comparative control in other phagocytosis assays.

Human DMs express CASR receptors

Having established a possible role for CASRs in immune recognition of *C. sordellii*, experiments were conducted to determine their expression levels in DMs using semi-quantitative 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 CASR receptors SR-AI/II, MARCO, SRCL, and SCARA-5. SRCL is an endothelial CASR that has also been identified on alveolar macrophages (42). SCARA5 was recently characterized in murine epithelial cells and was found to have similar binding properties to other CASR receptors (27). However,

functional roles in microbial clearance for either SRCL or SCARA5 receptors have not been identified for human phagocytes. The expression of two CBSRs implicated in binding bacteria, CD36 and CD163, was also evaluated (14, 20). These experiments revealed expression of multiple SRs (**Fig. 1B**). Of the CASRs, the expression of SR-AI/II was greatest, being significantly higher than SCARA-5, which was only minimally expressed. SR-AI/II transcripts were not significantly greater than mRNA levels of MARCO. We also found a significantly higher expression of CD163, when compared with CD36.

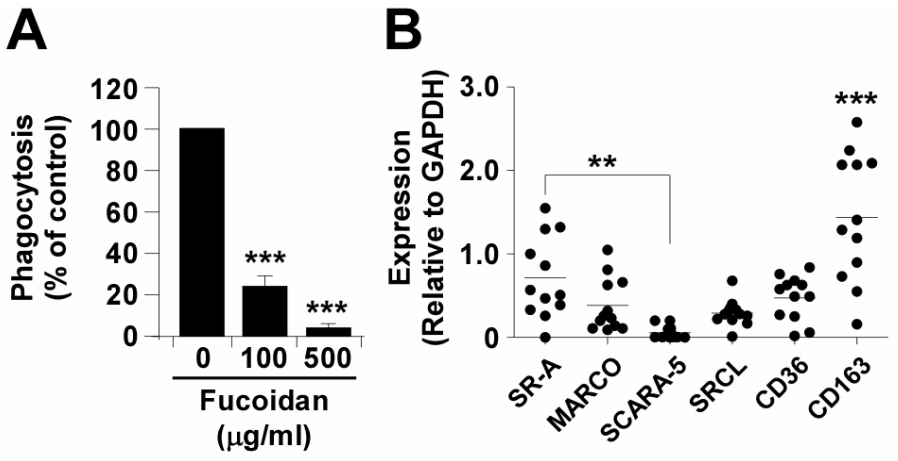

Figure 1. Evidence for functional scavenger receptors in human DMs. A, Human DMs were pretreated for 30 min with the scavenger receptor blocker fucoidan (in μg/ml) and phagocytosis of ^{FITC}*C. sordellii* quantified as detailed in Materials and Methods. Data are representative from three independent experiments performed in octuplet with similar results. Results are expressed as a percentage of phagocytosis relative to untreated cells. ***, $P < 0.001$ vs. untreated cells by ANOVA. **B**, Semi-quantitative real-time PCR performed to measure expression levels of scavenger receptors in human DMs, relative to GAPDH, described in Materials and Methods. Data points represent the mean result from 12 separate individuals performed in triplicate. **, $P < 0.01$; ***, $P < 0.001$ comparing CD163 against each of the other receptors (ANOVA).

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 pre-incubated with dextran sulfate (100 µg/ml), there was an 87.3 $\pm$ 6.1% decrease in phagocytic ability, while the negative control (but structurally similar) agent chondroitan sulfate (100 µg/ml) had no effect (**Fig. 2A**). Another classical CASR antagonist, Poly(I), suppressed phagocytosis by 48.5 $\pm$ 6.2% compared to vehicle treatment, while 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). While 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 identified a minor role for the mannose receptor in the macrophage phagocytosis of *C. perfringens* (35). However, we found no role for this receptor in the phagocytosis of *C. sordellii* when human DMs were pre-incubated (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).

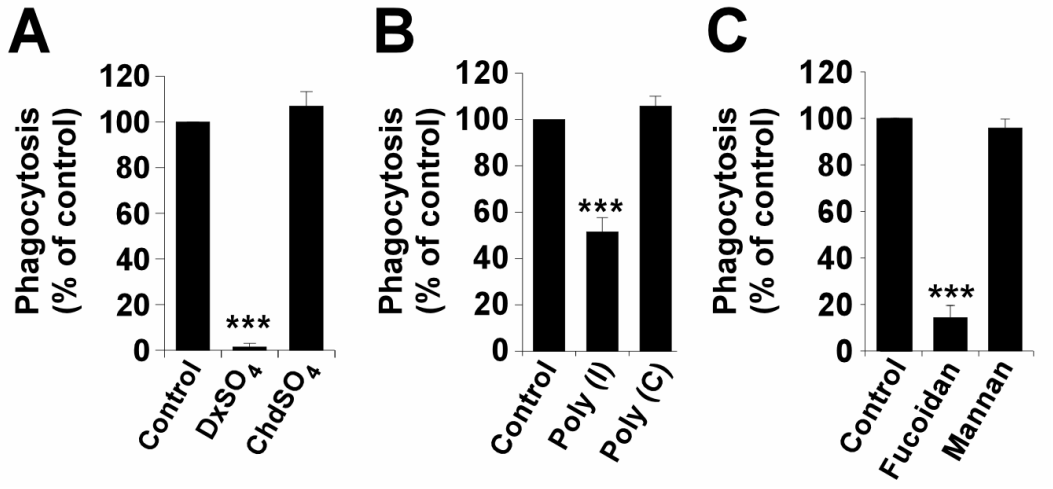

Figure 2. Non-selective class A scavenger blockers inhibit phagocytosis of *C. sordellii,* while mannose receptor blockers have no effect. A, human DMs were pretreated for 30 min with the CASR blocking agent dextran sulfate ($DxSO_4$, 100 μg/ml) or the negative control agent chondroitan sulfate ($ChdSO_4$, 100 μg/ml) 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 for 30 min with the CASR blocking agent Poly(I) (200 μg/ml) or the negative control Poly(C) (200 μg/ml) and the phagocytosis of ^{FITC}*C. sordellii* was quantified by fluorometry after 180 min as detailed in *Materials and Methods*. **C**, human DMs were pretreated with fucoidan (500 μg/ml) or the mannose receptor blocker, mannan (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*. Data are representative from three independent experiments performed in octuplet with similar results. Results are expressed as a percentage of relative to untreated cells. ***, $P < 0.001$ vs. control as determined by ANOVA.

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) are primarily responsible for internalizing *C. sordellii*, a previously published pharmacological approach to selectively antagonize these receptors was employed (39). Cells were preincubated (30 min) either with recombinant, mouse sMARCO or with the SR-AI/II-selective agent AcLDL (39). AcLDL had no effect on *C. sordellii* phagocytosis, while sMARCO inhibited phagocytosis by 79.5 $\pm$ 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 (14, 20, 45), we preincubated DMs with monoclonal antibodies 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 antibody had no effect (**Fig. 3B**). Thus, CD36 appears to play a minor role in phagocytosing *C. sordellii*.

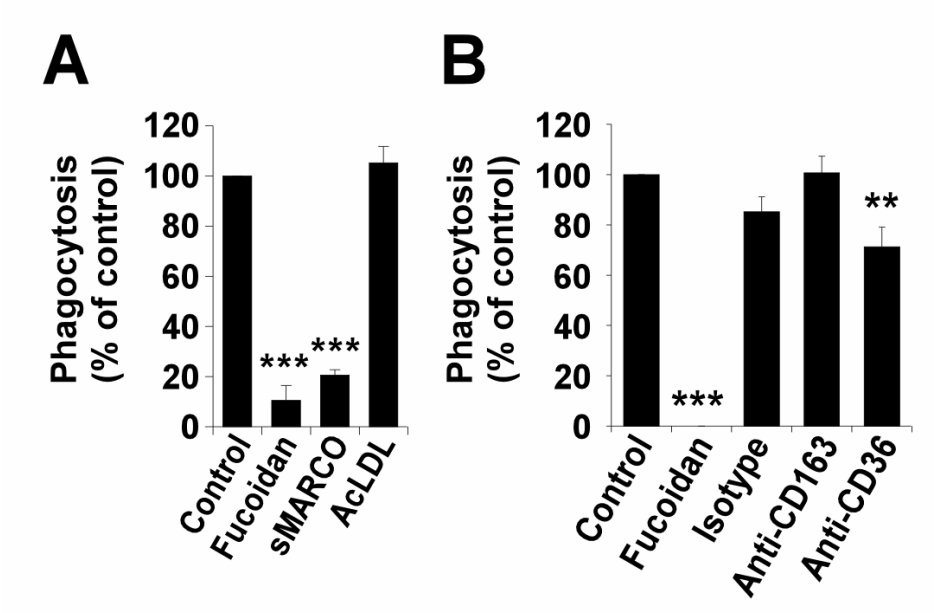

Figure 3. Soluble MARCO and anti-CD36 IgG prevent the phagocytosis of *C. sordellii* by human DMs. **A**, Human DMs were pretreated with sMARCO (100 µg/ml) or the SR-AI/II-binding agent acetylated low density lipoprotein (AcLDL; 100 µg/ml) for 30 min and the phagocytosis of ^{FITC}*C. sordellii* was 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 CD163 (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. **, $P < 0.01$ and ***, $P < 0.001$ vs. untreated cells.

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 to 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 than in WT cells (*P<0.05*).

These data suggest 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 MARCO. Real-time PCR (**Fig. 4B**) did not detect any significant compensatory increase in mRNA expression of SRA-I/II.

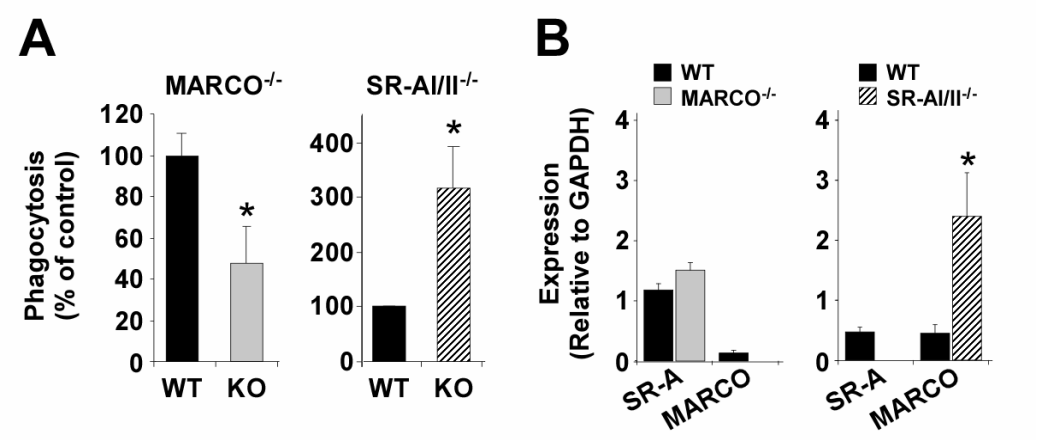

Figure 4. MARCO is more important than SR-AI/II in the phagocytosis of *C. sordellii*. **A**, Left panel, peritoneal macrophages from either wild-type (WT) BALB/c mice or MARCO$^{-/-}$ mice were allowed to phagocytose ^{FITC}*C. sordellii* for 3 hr. Right panel, peritoneal macrophages from either wild-type (WT) 129/SvJ mice or SR-AI/II$^{-/-}$ mice were allowed to phagocytose ^{FITC}*C. sordellii* for 3 hr. The phagocytosis of ^{FITC}*C. sordellii* was quantified by fluorometry as detailed in *Materials and Methods*. KO, knockout cells. **P* <0.05 vs. WT by Student *t*-test. Results are a mean ± SEM of 3-5 independent experiments performed in octuplet. **B**, mRNA expression of SR-AI/II or MARCO in mouse peritoneal macrophages was determined by semi-quantitative real-time PCR as detailed in *Materials and Methods*. Left panel, mRNA levels were compared between WT BALB/c peritoneal macrophages (black bars) or MARCO$^{-/-}$ cells (gray bars). Right panel, mRNA levels were compared between WT 129/SvJ peritoneal macrophages (black bars) or SR-AI/II$^{-/-}$ cells (striped bars). Data are normalized to GAPDH expression and represent the mean ± SEM of 8 mouse samples per assay. **P* < 0.05 vs. WT cells by Student *t*-test.

MARCO deficient mice have enhanced susceptibility to C. sordellii infection

Because the above *in vitro* experiments with mouse PMs and human DMs suggested that MARCO is an important immunosurveillance receptor used by FRT macrophages to defend the host against *C. sordellii*, we tested its importance, in this context, *in vivo*. Wild-type or MARCO$^{-/-}$ mice were inoculated intrauterine with the virulent *C. sordellii* strain ATCC9714. Using an inoculum of 1 x 10^4 CFU per mouse, only 15% of infected WT BALB/c mice died as a result of infection (**Fig. 5A**). In contrast, MARCO$^{-/-}$ mice were significantly more susceptible to death from infection, with a 3-fold higher mortality of 45%

by 7 days post-infection (**Fig. 5A**, n = 20 mice per group, $P = 0.04$ by Mantel-Cox log-rank test). In contrast, SR-AI/II$^{-/-}$ mice were not more susceptible to death from intrauterine *C. sordellii* infection compared to WT 129/SvJ mice (**Fig. 5C**).

MARCO is important for clearance of bacteria from the infected uterus

We questioned whether the increased mortality observed in MARCO$^{-/-}$ mice correlated with an impaired capacity to eliminate bacteria from the infected uterus. To address this question, mice were infected intrauterine with *C. sordellii* and the organ bacterial load was determined by anaerobically culturing tissue homogenates harvested at 24 hr post infection. As illustrated (**Fig. 5B**), there was a significantly greater bacterial load in the uterus 24 hr after infection in the MARCO$^{-/-}$ mice compared to WT animals. To address dissemination of infection from the uterus, spleens were harvested 24 hr following inoculation and bacterial burdens were determined for both WT and MARCO$^{-/-}$ mice. Although a greater number of MARCO-deficient mice had evidence of splenic dissemination compared to wild type mice (3 of 10 MARCO-deficient mice vs. 1 of 10 wild type mice), neither this outcome nor the mean bacterial loads per spleen were significantly different between the two groups (data not shown). We have not detected differences in local clearance of *C. sordellii* from the uterus or dissemination to the spleen between WT or SR-AI/II$^{-/-}$ mice (data not shown).

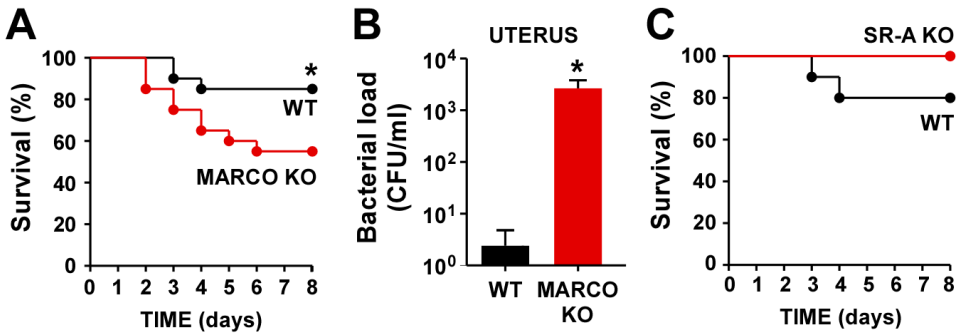

Figure 5. MARCO is important to host defense against intrauterine *C. sordellii* infection. A, Wild-type (WT) or MARCO knockout (KO) mice (n=20 per group) were infected by intrauterine injection with *C. sordellii* ATCC9714 ($1x10^4$ CFU per mouse) and survival was recorded. **P* < 0.05 vs. WT survival. **B**, The bacterial load of *C. sordellii* was determined in uterine homogenates 24 hrs after infection as described in the *Materials and Methods*. Shown are the mean ± SEM of 5 mice per group from one of two independent experiments showing similar results. **P* < 0.05 vs. WT. CFU, colony-forming units. **C**, Wild-type (WT) or SR-A knockout (KO) mice (n=10 per group) were infected by intrauterine injection with *C. sordellii* ATCC9714 ($1x10^4$ CFU per mouse) and survival was recorded. Differences were not significant.

Chapter 5: Discussion

These studies newly define a potentially important role for MARCO receptors in the innate host defense against invasive *C. sordellii* infections of the FRT. Infections caused by toxigenic clostridia are emerging as important challenges to human health. While 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 (2, 24, 25) 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 antigens 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 NFκB (1). 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 (35). Phagocytosis of *C. perfringens* was impaired in those studies using the non-selective CASR blocking agent fucoidan (35). 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 (35).

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 (35), 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 a real-time PCR based approach, we identified transcription of the *MSR1* 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 non-hematopoeitic cells (*e.g.*, endothelial cells) (4, 42). We also investigated whether SCARA-5 was expressed by human DMs, as this receptor has not been well characterized in human cells and tissues (27). However, we did not observe significant expression of this receptor.

To confirm the results obtained with fucoidan, we utilized pairs of non-selective CASR blocking agents and structurally matched, negative control compounds that do not bind to CASRs (*e.g.*, Poly(I)-Poly(C) and dextran sulfate-chondroitan sulfate). Indeed, phagocytosis was inhibited by both dextran sulfate and poly(I) but not by the negative control agents chondroitan sulfate or poly(C).

A study by Laskarin *et al.* suggested the mannose receptor is a functional receptor on decidual mononuclear cells (29). The exact role of the mannose receptor in phagocytosis is uncertain with conflicting biochemical and genetic studies (19). Due to its decreased

expression during inflammation (16) it has been suggested the mannose receptor may be more important for tissue homeostasis than for host defense (19). However, taking into consideration this information and the previously mentioned studies by O'Brien and Melville, 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.

TLR2 has been shown to play a role in bacterial uptake by both macrophages (46) and mouse trophoblast giant cells (48). Because of this potential role for TLRs in *C. sordellii* infections, we also performed phagocytosis assays with human DMs using a TLR2-blocking IgG. When pretreating the cells at 50 or 100 µg/ml, we found no inhibition of phagocytosis when compared to an isotype control IgG (data not shown). This suggests TLR2 is not involved in the internalization of *C. sordellii*.

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 (38, 39). We initially incubated human DMs with either AcLDL, a specific antagonist of SR-AI/II receptors (39), or sMARCO, a specific inhibitor of MARCO-dependent phagocytosis (39, 41). 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, though on a different genetic background (28). 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 than WT macrophages (**Fig. 4**), providing more evidence that MARCO expression is up-regulated in mouse cells lacking SR-AI/II. These data were limited by the fact that mRNA transcripts 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 antibody (28).

While our *in vivo* data provide novel and correlative support for our *in vitro* results that MARCO is an important component of the innate defense against *C. sordellii* infections, it does not prove that decidual macrophages are the only cells involved in such uterine infections. To address this, we used the human uterine epithelial cell (UEC) line, RL95-2 (purchased from the ATCC), in phagocytosis assays in the same manner as with decidual macrophages to determine if they were able to phagocytose or otherwise internalize *C. sordellii*. We did not observe any significant intracellular fluorescence in these UECs (data not shown), indicating these cells do not avidly take up *C. sordellii in vitro* or *in vivo.*

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 and B**). As expected, we did not see any

enhanced susceptibility to infection in SR-AI/II null mice (**Fig. 5C**). We have also not observed differences in bacterial clearance from the uterus comparing WT and SR-AI/II null mice (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 (8). 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 (8). 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 (non-sporulating) 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 (30). 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 monoclonal antibodies 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 SRs we examined by semi-quantitative real time PCR (**Fig. 1B**)). The CD163 receptor is involved primarily in the clearance of hemoglobin-haptoglobin complexes (31). It has been implicated in anti-inflammatory responses and is a surface marker of alternative (M2) macrophage activation (31). Thus, our finding of CD163 expression by human DMs provides further support to other studies demonstrating that DMs exhibit features of alternative activation (17, 23). The implications of this for bacterial infections in the postpartum or post-abortion period are unclear.

In summary, we newly characterize SR 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. DMs are of paramount importance in host defense against bacterial infection and also regulate immunity at the fetal/maternal interface to induce tolerance of the fetus by the mother (26). Few studies have specifically examined host-microbial interactions between invading bacterial pathogens and FRT macrophages. 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 non-sexually transmitted infections.

References

1. **Aldape, M. J., A. E. Bryant, E. J. Katahira, A. M. Hajjar, S. M. Finegold, Y. Ma, and D. L. Stevens.** 2009. Innate immune recognition of, and response to, *Clostridium sordellii*. Anaerobe.
2. **Aldape, M. J., A. E. Bryant, and D. L. Stevens.** 2006. *Clostridium sordellii* infection: epidemiology, clinical findings, and current perspectives on diagnosis and treatment. Clin Infect Dis **43:**1436-46.
3. **Anonymous.** March 2008, posting date. Information about *Clostridium Sordellii*. Centers for Disease Control and Prevention. http://www.cdc.gov/ncidod/dhqp/id_Csordellii.html.
4. **Areschoug, T., and S. Gordon.** 2009. Scavenger receptors: role in innate immunity and microbial pathogenesis. Cell Microbiol **11:**1160-9.
5. **Aronoff, D. M., and J. D. Ballard.** 2009. Clostridium sordellii toxic shock syndrome. Lancet Infect Dis **9:**725-6.
6. **Aronoff, D. M., C. Canetti, and M. Peters-Golden.** 2004. Prostaglandin E2 inhibits alveolar macrophage phagocytosis through an E-prostanoid 2 receptor-mediated increase in intracellular cyclic AMP. J Immunol **173:**559-65.
7. **Aronoff, D. M., Y. Hao, J. Chung, N. Coleman, C. Lewis, C. M. Peres, C. H. Serezani, G. H. Chen, N. Flamand, T. G. Brock, and M. Peters-Golden.** 2008. Misoprostol impairs female reproductive tract innate immunity against *Clostridium sordellii*. J Immunol **180:**8222-30.
8. **Arredouani, M., Z. Yang, Y. Ning, G. Qin, R. Soininen, K. Tryggvason, and L. Kobzik.** 2004. The scavenger receptor MARCO is required for lung defense against pneumococcal pneumonia and inhaled particles. J Exp Med **200:**267-72.
9. **Arredouani, M. S., F. Franco, A. Imrich, A. Fedulov, X. Lu, D. Perkins, R. Soininen, K. Tryggvason, S. D. Shapiro, and L. Kobzik.** 2007. Scavenger Receptors SR-AI/II and MARCO limit pulmonary dendritic cell migration and allergic airway inflammation. J Immunol **178:**5912-20.
10. **Arredouani, M. S., A. Palecanda, H. Koziel, Y. C. Huang, A. Imrich, T. H. Sulahian, Y. Y. Ning, Z. Yang, T. Pikkarainen, M. Sankala, S. O. Vargas, M. Takeya, K. Tryggvason, and L. Kobzik.** 2005. MARCO is the major binding receptor for unopsonized particles and bacteria on human alveolar macrophages. J Immunol **175:**6058-64.
11. **Arredouani, M. S., Z. Yang, A. Imrich, Y. Ning, G. Qin, and L. Kobzik.** 2006. The macrophage scavenger receptor SR-AI/II and lung defense against pneumococci and particles. Am J Respir Cell Mol Biol **35:**474-8.
12. **Bailie, M. B., T. J. Standiford, L. L. Laichalk, M. J. Coffey, R. Strieter, and M. Peters-Golden.** 1996. Leukotriene-deficient mice manifest enhanced lethality from *Klebsiella pneumonia* in association with decreased alveolar macrophage phagocytic and bactericidal activities. J Immunol **157:**5221-4.
13. **Ballinger, M. N., R. Paine, 3rd, C. H. Serezani, D. M. Aronoff, E. S. Choi, T. J. Standiford, G. B. Toews, and B. B. Moore.** 2006. Role of granulocyte macrophage colony-stimulating factor during gram-negative lung infection with *Pseudomonas aeruginosa*. Am J Respir Cell Mol Biol **34:**766-74.
14. **Baranova, I. N., R. Kurlander, A. V. Bocharov, T. G. Vishnyakova, Z. Chen, A. T. Remaley, G. Csako, A. P. Patterson, and T. L. Eggerman.** 2008. Role of human

CD36 in bacterial recognition, phagocytosis, and pathogen-induced JNK-mediated signaling. J Immunol **181:**7147-56.

15. **Bermudez, L. E., A. Parker, and J. R. Goodman.** 1997. Growth within macrophages increases the efficiency of *Mycobacterium avium* in invading other macrophages by a complement receptor-independent pathway. Infect Immun **65:**1916-25.
16. **Chroneos, Z., and V. L. Shepherd.** 1995. Differential regulation of the mannose and SP-A receptors on macrophages. Am J Physiol **269:**L721-6.
17. **Cupurdija, K., D. Azzola, U. Hainz, A. Gratchev, A. Heitger, O. Takikawa, S. Goerdt, R. Wintersteiger, G. Dohr, and P. Sedlmayr.** 2004. Macrophages of human first trimester decidua express markers associated to alternative activation. Am J Reprod Immunol **51:**117-22.
18. **Dunne, D. W., D. Resnick, J. Greenberg, M. Krieger, and K. A. Joiner.** 1994. The type I macrophage scavenger receptor binds to gram-positive bacteria and recognizes lipoteichoic acid. Proc Natl Acad Sci U S A **91:**1863-7.
19. **Ernst, J. D., and O. Stendahl (ed.).** 2006. Phagocytosis of Bacteria and Bacterial Pathogenicity, vol. Cambridge University Press, New York, Cambridge.
20. **Fabriek, B. O., R. van Bruggen, D. M. Deng, A. J. Ligtenberg, K. Nazmi, K. Schornagel, R. P. Vloet, C. D. Dijkstra, and T. K. van den Berg.** 2009. The macrophage scavenger receptor CD163 functions as an innate immune sensor for bacteria. Blood **113:**887-92.
21. **Fischer, M., J. Bhatnagar, J. Guarner, S. Reagan, J. K. Hacker, S. H. Van Meter, V. Poukens, D. B. Whiteman, A. Iton, M. Cheung, D. E. Dassey, W. J. Shieh, and S. R. Zaki.** 2005. Fatal toxic shock syndrome associated with *Clostridium sordellii* after medical abortion. N Engl J Med **353:**2352-60.
22. **Greenberg, J. W., W. Fischer, and K. A. Joiner.** 1996. Influence of lipoteichoic acid structure on recognition by the macrophage scavenger receptor. Infect Immun **64:**3318-25.
23. **Gustafsson, C., J. Mjosberg, A. Matussek, R. Geffers, L. Matthiesen, G. Berg, S. Sharma, J. Buer, and J. Ernerudh.** 2008. Gene expression profiling of human decidual macrophages: evidence for immunosuppressive phenotype. PLoS One **3:**e2078.
24. **Hao, Y., T. Senn, S. O. J, V. B. Young, T. Thiele, G. Srinivas, S. K. Huang, and D. M. Aronoff.** 2009. Lethal toxin is a critical determinant of rapid mortality in rodent models of *Clostridium sordellii* endometritis. Anaerobe.
25. **Ho, C. S., J. Bhatnagar, A. L. Cohen, J. K. Hacker, S. B. Zane, S. Reagan, M. Fischer, W. J. Shieh, J. Guarner, S. Ahmad, S. R. Zaki, and L. C. McDonald.** 2009. Undiagnosed cases of fatal Clostridium-associated toxic shock in Californian women of childbearing age. Am J Obstet Gynecol **201:**459 e1-7.
26. **Hunt, J. S.** 1990. Current topic: the role of macrophages in the uterine response to pregnancy. Placenta **11:**467-75.
27. **Jiang, Y., P. Oliver, K. E. Davies, and N. Platt.** 2006. Identification and characterization of murine SCARA5, a novel class A scavenger receptor that is expressed by populations of epithelial cells. J Biol Chem **281:**11834-45.

28. **Jozefowski, S., M. Arredouani, T. Sulahian, and L. Kobzik.** 2005. Disparate regulation and function of the class A scavenger receptors SR-AI/II and MARCO. J Immunol **175:**8032-41.
29. **Laskarin, G., K. Cupurdija, V. S. Tokmadzic, D. Dorcic, J. Dupor, K. Juretic, N. Strbo, T. B. Crncic, F. Marchezi, P. Allavena, A. Mantovani, L. Randic, and D. Rukavina.** 2005. The presence of functional mannose receptor on macrophages at the maternal-fetal interface. Hum Reprod **20:**1057-66.
30. **Matsumoto, A., M. Naito, H. Itakura, S. Ikemoto, H. Asaoka, I. Hayakawa, H. Kanamori, H. Aburatani, F. Takaku, H. Suzuki, and et al.** 1990. Human macrophage scavenger receptors: primary structure, expression, and localization in atherosclerotic lesions. Proc Natl Acad Sci U S A **87:**9133-7.
31. **Moestrup, S. K., and H. J. Moller.** 2004. CD163: a regulated hemoglobin scavenger receptor with a role in the anti-inflammatory response. Ann Med **36:**347-54.
32. **Murphy, J. E., P. R. Tedbury, S. Homer-Vanniasinkam, J. H. Walker, and S. Ponnambalam.** 2005. Biochemistry and cell biology of mammalian scavenger receptors. Atherosclerosis **182:**1-15.
33. **Mwaikambo, B. R., F. Sennlaub, H. Ong, S. Chemtob, and P. Hardy.** 2006. Activation of CD36 inhibits and induces regression of inflammatory corneal neovascularization. Invest Ophthalmol Vis Sci **47:**4356-64.
34. **O'Brien, D. K., and S. B. Melville.** 2004. Effects of *Clostridium perfringens* alpha-toxin (PLC) and perfringolysin O (PFO) on cytotoxicity to macrophages, on escape from the phagosomes of macrophages, and on persistence of *C. perfringens* in host tissues. Infect Immun **72:**5204-15.
35. **O'Brien, D. K., and S. B. Melville.** 2003. Multiple effects on *Clostridium perfringens* binding, uptake and trafficking to lysosomes by inhibitors of macrophage phagocytosis receptors. Microbiology **149:**1377-86.
36. **Palecanda, A., J. Paulauskis, E. Al-Mutairi, A. Imrich, G. Qin, H. Suzuki, T. Kodama, K. Tryggvason, H. Koziel, and L. Kobzik.** 1999. Role of the scavenger receptor MARCO in alveolar macrophage binding of unopsonized environmental particles. J Exp Med **189:**1497-506.
37. **Peters-Golden, M., R. W. McNish, R. Hyzy, C. Shelly, and G. B. Toews.** 1990. Alterations in the pattern of arachidonate metabolism accompany rat macrophage differentiation in the lung. J Immunol **144:**263-70.
38. **Pluddemann, A., S. Mukhopadhyay, and S. Gordon.** 2006. The interaction of macrophage receptors with bacterial ligands. Expert Rev Mol Med **8:**1-25.
39. **Pluddemann, A., S. Mukhopadhyay, M. Sankala, S. Savino, M. Pizza, R. Rappuoli, K. Tryggvason, and S. Gordon.** 2009. SR-A, MARCO and TLRs Differentially Recognise Selected Surface Proteins from Neisseria meningitidis: an Example of Fine Specificity in Microbial Ligand Recognition by Innate Immune Receptors. Journal of Innate Immunity **1:**153-163.
40. **Rood, J. I., McClane B.A., Songer J.G., Titball R.W.** 1997. The Clostridia: Molecular Biology and Pathogenesis, vol. Academic Press, San Diego.
41. **Sankala, M., A. Brannstrom, T. Schulthess, U. Bergmann, E. Morgunova, J. Engel, K. Tryggvason, and T. Pikkarainen.** 2002. Characterization of recombinant soluble macrophage scavenger receptor MARCO. J Biol Chem **277:**33378-85.

42. **Selman, L., K. Skjodt, O. Nielsen, C. Floridon, U. Holmskov, and S. Hansen.** 2008. Expression and tissue localization of collectin placenta 1 (CL-P1, SRCL) in human tissues. Mol Immunol **45:**3278-88.
43. **Sinave, C., G. Le Templier, D. Blouin, F. Leveille, and E. Deland.** 2002. Toxic shock syndrome due to *Clostridium sordellii*: a dramatic postpartum and postabortion disease. Clin Infect Dis **35:**1441-3.
44. **Singh, U., G. Nicholson, B. C. Urban, I. L. Sargent, U. Kishore, and A. L. Bernal.** 2005. Immunological properties of human decidual macrophages--a possible role in intrauterine immunity. Reproduction **129:**631-7.
45. **Stuart, L. M., J. Deng, J. M. Silver, K. Takahashi, A. A. Tseng, E. J. Hennessy, R. A. Ezekowitz, and K. J. Moore.** 2005. Response to *Staphylococcus aureus* requires CD36-mediated phagocytosis triggered by the COOH-terminal cytoplasmic domain. J Cell Biol **170:**477-85.
46. **Underhill, D. M., A. Ozinsky, K. D. Smith, and A. Aderem.** 1999. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. Proc Natl Acad Sci U S A **96:**14459-63.
47. **Vince, G. S., P. M. Starkey, M. C. Jackson, I. L. Sargent, and C. W. Redman.** 1990. Flow cytometric characterisation of cell populations in human pregnancy decidua and isolation of decidual macrophages. J Immunol Methods **132:**181-9.
48. **Watanabe, K., E. K. Shin, M. Hashino, M. Tachibana, and M. Watarai.** Toll-like receptor 2 and class B scavenger receptor type I are required for bacterial uptake by trophoblast giant cells. Mol Immunol.
49. **Wilson, M.** 2005. Microbial inhabitants of humans: their ecology and role in health and disease, First ed, vol. Cambridge University Press, Cambridge.

APPENDICES

Appendix A: Approval Letter for UCUCA application #10044

From: eSiriusWebServer <esirius-ucuca@med.umich.edu>
To: <daronoff@umich.edu>
Date: 5/14/2008 12:26 PM
Subject: Approval Letter for UCUCA application #10044

CC: <oconnord@umich.edu>

PI: Aronoff, David M
Protocol Number: #10044
Approval Period: 05/14/2008 - 05/14/2011
Funding Agency: National Institutes of Health
Title: Role of Misoprostol in Clostridium sordellii Endometritis After Medical Abortion
DRDA Number:

Date: 05/14/2008

Dear Principal Investigator,

The University of Michigan Committee on Use and Care of Animals (UCUCA) has reviewed your application to use vertebrate animals (Application #10044). This project has been approved. The proposed animal use procedures are in compliance with University guidelines, State and Federal regulations, and the standards of the "Guide for the Care and Use of Laboratory Animals."

When communicating with the UCUCA Office please refer to the Approval Number #10044. The approval number must accompany all requisitions for animals and pharmaceuticals.

The approval date is 05/14/2008. The approval period is for three years from this date. However, the United States Department of Agriculture (USDA) requires an annual review of applications to use animals. Therefore, each year of this application prior to the anniversary of its approval date, you will be notified via email to submit a short annual review. Your continued animal use approval is contingent upon the completion and return of this annual review. You will also be notified 120 days prior to the expiration of the approval period so that your renewal application can be prepared, submitted and reviewed in a timely manner in the eSirius program and an interruption in the approval status of this project avoided.

UCUCA approval must be obtained prior to changes from what is originally stated in the protocol. An amendment must be submitted to the UCUCA for review and approved prior to the implementation of the proposed change.

The University's Animal Welfare Assurance Number on file with the NIH Office of Laboratory Animal Welfare (OLAW) is A3114-01, and most recent date of accreditation by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC, Intl.) is November 14, 2005.

If you receive news media inquiries concerning any aspect of animal use or care in this project, please contact James Erickson, News and Information Services, 647-1842. If you have security concerns regarding the animals or animal facilities, contact Bill Bess, Director of Public Safety, 763-3434.

Sincerely,

Susan Stern, MD
Associate Professor, Emergency Medicine
Chairperson, University Committee on Use and Care of Animals

Appendix B: IRB approval

https://eresearch.umich.edu/eresearch/Doc/0/QUO1NE4HFP7K9CHPJ2KILMP4F2/fromString.html
Medical School Institutional Review Board (IRBMED) • Argus I Building, 517 W. William, Ann Arbor, MI 48103-4943 • phone (734) 763 4768 • fax (734)
763 9603 • irbmed@umich.edu

To: Dr. David Aronoff

From:
Michael Geisser
John Weg

Cc:
David Aronoff
Laura Castleman
Rita Loch-Caruso
Jason Bell
Lisa Harris

Subject: Scheduled Continuing Review [CR00012813] Approved for [HUM00008514]

SUBMISSION INFORMATION:
Study Title: Regulation of innate immunity in the female reproductive tract by misoprostol: impact on Clostridium sordellii infection following abortion
Full Study Title (if applicable):
Study eResearch ID: HUM00008514
SCR eResearch ID: CR00012813
SCR Title: HUM00008514_Continuing Review - Tue Sep 8 09:44:57 EDT 2009
Date of this Notification from IRB:10/26/2009
Date Approval for this SCR: 10/24/2009
Current IRB Approval Period: 10/24/2009 - 10/23/2010
Expiration Date: Approval for this expires **at 11:59 p.m. on 10/23/2010**
UM Federalwide Assurance:FWA00004969 expiring on 11/17/2011
OHRP IRB Registration Number(s): IRB00001996

NOTICE OF IRB APPROVAL AND CONDITIONS:

https://eresearch.umich.edu/eresearch/Doc/0/QUO1NE4HFP7K9CHPJ2KILMP4F2/fromString.html
The IRBMED has reviewed and approved the scheduled continuing review (SCR) submitted for the study referenced above. The IRB determined that the proposed research continues to conform with applicable guidelines, State and federal regulations, and the University of Michigan's Federalwide Assurance (FWA) with the Department of Health and Human Services (HHS).

You must conduct this study in accordance with the description and information provided in the approved application and associated documents.

APPROVAL PERIOD AND EXPIRATION DATE:
The updated approval period for this study is listed above. Please note the expiration date. If the approval lapses, you may not conduct work on this study until appropriate approval has been reestablished, except as necessary to eliminate apparent immediate hazards to research subjects or others. Should the latter occur, you must notify the IRB Office as soon as possible.

IMPORTANT REMINDERS AND ADDITIONAL INFORMATION FOR INVESTIGATORS
APPROVED STUDY DOCUMENTS:
You must use any date-stamped versions of recruitment materials and informed consent documents available in the eResearch workspace (referenced above). Date-stamped materials are available in the "Currently Approved Documents" section on the "Documents" tab. In accordance with 45 CFR 46.111 and IRB practice, consent document(s) and process are considered as part of Continuing Review to ensure accuracy and completeness. The dates on the consent documents, if applicable, have been updated to reflect the date of Continuing Review approval.

RENEWAL/TERMINATION:
At least two months prior to the expiration date, you should submit a continuing review application either to renew or terminate the study. Failure to allow sufficient time for IRB review may result in a lapse of approval that may also affect any funding associated with the study.

AMENDMENTS:
All proposed changes to the study (e.g., personnel, procedures, or documents), must be approved in
advance by the IRB through the amendment process, except as necessary to eliminate apparent immediate hazards to research subjects or others. Should the latter occur, you must notify the IRB Office as soon as possible.

AEs/ORIOs:
You must continue to inform the IRB of all unanticipated events, adverse events (AEs), and other reportable information and occurrences (ORIOs). These include but are not limited to events and/or information that may have physical, psychological, social, legal, or economic impact on the research subjects or others.

Investigators and research staff are responsible for reporting information concerning the approved research to the IRB in a timely fashion, understanding and adhering to the reporting guidance (http://

www.med.umich.edu/irbmed/ae_orio/index.htm), and not implementing any changes to the research without IRB approval of the change via an amendment submission. When changes are necessary to eliminate apparent immediate hazards to the subject, implement the change and report via an ORIO and/or amendment submission within 7 days after the action is taken. This includes all information with the potential to impact the risk or benefit assessments of the research.

SUBMITTING VIA eRESEARCH:
https://eresearch.umich.edu/eresearch/Doc/0/QUO1NE4HFP7K9CHPJ2KILMP4F2/fromString.html (2 of 3)8/11/2010 2:51:29 PM
https://eresearch.umich.edu/eresearch/Doc/0/QUO1NE4HFP7K9CHPJ2KILMP4F2/fromString.html
You can access the online forms for continuing review, amendments, and AE/ORIO reporting in the eResearch workspace for this approved study, referenced above.

MORE INFORMATION:
You can find additional information about UM's Human Research Protection Program (HRPP) in the Operations Manual and other documents available at: www.research.umich.edu/hrpp.

Michael Geisser
Co-chair, IRBMED
John Weg
Co-chair, IRBMED
https://eresearch.umich.edu/eresearch/Doc/0/QUO1NE4HFP7K9CHPJ2KILMP4F2/fromString.html (3 of 3)8/11/2010 2:51:29 PM