A naturally occurring transcript variant of MARCO reveals the SRR domain is critical for function

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Macrophage receptor with collagenous structure (MARCO) is a class A scavenger receptor (cA-SR) that recognizes and phagocytoses a wide variety of pathogens. Most cA-SRs that contain a C-terminal scavenger receptor cysteine-rich (SRCR) domain use the proximal collagenous domain to bind ligands. In contrast, the role of the SRCR domain of MARCO in phagocytosis, adhesion and pro-inflammatory signaling is less clear. The discovery of a naturally occurring transcript variant lacking the SRCR domain, MARCOII, provided the opportunity to study the role of the SRCR domain of MARCO. We tested whether the SRCR domain is required for ligand binding, promoting downstream signaling and enhancing cellular adhesion. Unlike cells expressing full-length MARCO, ligand binding was abolished in MARCOII-expressing cells. Furthermore, co-expression of MARCO and MARCOII impaired phagocytic function, indicating that MARCOII acts as a dominant-negative variant. Unlike MARCO, expression of MARCOII did not enhance Toll-like receptor 2 (TLR2)-mediated pro-inflammatory signaling in response to bacterial stimulation. MARCO-expressing cells were more adherent and exhibited a dendritic-like phenotype, whereas MARCOII-expressing cells were less adherent and did not exhibit changes in morphology. These data suggest the SRCR domain of MARCO is the key domain in modulating ligand binding, enhancing downstream pro-inflammatory signaling and MARCO-mediated cellular adhesion.

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The class A scavenger receptors (cA-SRs) are a diverse group of multifunctional pattern recognition receptors that are required for antibacterial immunity, adhesion, motility and homeostasis.1 cA-SRs bind a wide array of polyanionic ligands, including, but not limited to, modified low-density lipoproteins, inorganic particulates and bacterial ligands, such as lipopolysaccharide and lipoteichoic acid.2 The ‘ligand-promiscuous’ cA-SRs have a major role in recognizing conserved pathogen-associated molecular patterns, and, thus, are critical in anti-bacterial immunity.3 Despite belonging to a single class, the ligand binding site(s) of the cA-SRs are not identical and remain controversial. The cA-SRs scavenger receptor class A (SRA), macrophage receptor with collagenous structure (MARCO) and scavenger receptor class A, member 5 (SCARA5) all contain a C-terminal scavenger receptor cysteine-rich (SRCR) domain. SRCR domains are found in many protein families and are often involved in ligand binding, but the exact role(s) of the SRCR domain within the cA-SRs remains enigmatic.4 The SRCR domain of MARCO has been proposed as the primary ligand binding site and was shown to bind ligands directly via two highly conserved arginine residues, termed the RxR motif.5,6 Although the SRCR domains of SRA, SCARA5 and MARCO all exhibit a high degree of evolutionary conservation, SRA does not contain a RxR motif and has been shown to primarily bind ligands within the proximal collagenous domain.6,7 In addition to SRA, the collagenous domain of SCARA3 has also very recently been identified as a putative ligand binding site for bacteria and modified low-density lipoprotein.8 SRA has been shown to exist as multiple splice variants; full-length (SRA-I), lacking the SRCR domain (SRA-II) and a dominant-negative isoform trapped in the endoplasmic reticulum (SRA-III).9 Although the expression of SRA splice variants is differentially regulated, SRA-I/II have been shown to bind ligands with similar specificity and affinity, providing evidence that the SRCR domain is not required for ligand binding.10,11 Multiple splice variants of MARCO have been deposited to public databases, but have never been functionally characterized. Using publically available transcript databases, we have identified a naturally occurring splice variant of MARCO lacking the exons coding for the SRCR domain.12 To further understand the role of the SRCR domain of MARCO, we have functionally characterized this splice variant, herein referred to as MARCOII. To our knowledge, this is the first study to functionally characterize any splice variant of MARCO. We have shown that loss of the SRCR domain abrogates binding of ligands, diminishes the ability for MARCO to enhance...
pro-inflammatory signaling and abolishes MARCO-mediated cellular adhesion.

RESULTS

Identification, cloning and expression of alternatively spliced MARCO transcripts

In order to characterize the functional importance of the SRCR domain of MARCO, we cloned a transcript variant lacking the SRCR domain using the Aceview human 2010 transcript database (GenBank accession number CR603381), which we call MARCOII. The domain structure of MARCO consists of a N-terminal cytoplasmic domain (aa 1–50) followed by a transmembrane domain (aa 51–74), spacer domain (aa 75–149), collagenous domain (aa 150–419) and SRCR domain (aa 420–520). In comparison, the predicted structure of MARCOII was identical to full-length MARCO for the first four domains, but was considerably different in the SRCR domain. The SRCR domain of MARCOII contained only the first 8 residues followed by an out-of-frame region of 19 residues (Figure 1a).

A full-length complementary DNA (cDNA) clone (CS0DM004YJ08) was generously provided by Wu-Bo Li (Life Technologies, Frederick, MD, USA, Supplementary Figure 1). To validate the presence of MARCO transcript variants in primary human samples, we used PCR to amplify exons 16/17 (surrounding the putative SRCR domain) in human peripheral blood mononuclear cells (PBMCs) followed by gel electrophoresis analysis. As indicated by the presence of a small band (approximately 200 bp) in addition to a large band (approximately 400 bp) (Figure 1b), we confirmed the presence of MARCO transcript variant (MARCOII) with a truncation of the putative SRCR domain. To determine if expression of the MARCOII transcript could be induced, PBMCs were stimulated for 48 h with either phosphate-buffered saline (PBS) (unstimulated, US), lipopolysaccharide (LPS), interleukin-10 (IL-10) or interferon-gamma (IFN-γ) stimulated PBMCs. Primers surround exons 14/17. Although MARCO transcript was detected in all samples, the MARCOII transcript was not expressed in 2/4 donors. Sequences of both transcripts were excised, purified and sequenced to confirm the identity of both transcripts.

To better understand the functional properties of MARCOII as well as the importance of the SRCR domain, the MARCOII cDNA was...
subcloned into pcDNA3.1/Hygro(+). Expression of MARCOII was assessed in transiently transfected HEK 293 T cells first by western blot analysis using antibodies targeting the cytoplasmic domain and out-of-frame ‘SRCR region’ of MARCOII. MARCOII was shown to have comparable expression to full-length MARCO (Figure 2a). Next, we added a C-terminal myc tag to better compare surface expression of MARCO and MARCOII by immunofluorescence (IF) microscopy. In our transient transfection system, myc-MARCOII was shown to be expressed at the cell surface (Figures 2c–j) and at similar levels as full-length MARCO (Figure 2b). Myc-tagged constructs were used for subsequent IF experiments.

MARCOII shows abrogated ligand binding ability

We compared the ability of MARCO and MARCOII to bind polystyrene microspheres (500 nm), which were passively coated with maleylated bovine serum albumin (Mal-BSA; a previously confirmed MARCO ligand).² Transiently transfected HEK 293 T cells expressing MARCO showed a 300% increase in binding Mal-BSA-coated microspheres when compared with MARCOII-expressing cells (Figures 3a–i). Similarly, MARCO-transfected cells showed a 230% increase in internalization of Mal-BSA-coated microspheres when compared with MARCOII-expressing cells or empty vector control cells (Figure 3j). The same trend was observed for myc-tagged constructs, suggesting that the addition of a C-terminal myc tag does not interfere with ligand binding (data not shown). In order to confirm these quantitative data were due to microsphere binding to MARCO-expressing cells, we performed IF microscopy, (Figures 3a–h). This phenomenon was not observed in myc-MARCOII-expressing cells, further indicating that microsphere binding was dependent on the SRCR domain of MARCO.

To determine if expression of MARCOII can affect endogenous MARCO function, we transiently transfected a HEK 293 T cell line stably expressing MARCO with MARCOII and performed ligand-coated microsphere binding and uptake assays as above. This resulted in a 72% reduction in ligand binding and a 61% reduction in ligand internalization (Figures 3k and l). We used a similar approach to determine if expression of MARCO could rescue the function of MARCOII-expressing cells. Conversely, we transfected a stably expressing MARCO HEK 293 T cell line with MARCO and performed the above assay. We observed a 172% increase in ligand binding and a 218% increase in ligand internalization (Figures 3m and n). It has been shown that the collagenous domain of the cA-SRPs is critical for homotrimerization.¹¹ Given that MARCO and MARCOII share identical collagenous domains, we performed a co-immunoprecipitation and showed that MARCO and MARCOII can form heterotrimeric complexes in HEK 293 T cells (Figure 3o).

Taken together, these data demonstrate that the SRCR domain of MARCO is required to bind and internalize Mal-BSA-coated microspheres.

The SRCR domain of MARCO binds Streptococcus pneumoniae and enhances Toll-like receptor 2 (TLR2)/CD14-mediated nuclear factor (NF)-κB activity

In addition to our microsphere binding assay, we sought to determine whether the SRCR domain could directly bind Streptococcus pneumoniae, a pathogenic bacterium that we have previously shown is cleared from the murine nasopharynx in a MARCO-dependent manner.¹³ To do so, we created a recombinant, soluble SRCR trimer. Following incubation of the SRCR construct with BSA- or Mal-BSA-coated microspheres, analysis by flow cytometry confirmed the SRCR domain binds Mal-BSA-coated microspheres. In addition, we confirmed that the SRCR domain binds S. pneumoniae (Figure 4b).
Although MARCO has never been shown to directly signal in response to ligand binding, it has been shown to enhance TLR2/CD14 signaling in response to *S. pneumoniae*. We hypothesized that the SRCR domain of MARCO may be required to enhance the activation of other pattern recognition receptors such as TLR2. To test this, we used a NF-κB secreted embryonic alkaline phosphatase reporter assay to assess pro-inflammatory signals in response to *S. pneumoniae* stimulation. HEK 293 T cells transfected with MARCO, TLR2 and CD14 showed a significant increase in NF-κB activation when compared with TLR2 and CD14 alone (Figure 4a). Cells transfected with MARCOII, TLR2 and CD14 showed no significant change in NF-κB activation when compared with cells transfected with TLR2 and CD14 alone (Figure 4a). This suggests that the SRCR domain of MARCO is critical for enhancing NF-κB activity via TLR2.

To assess whether the soluble SRCR trimer alone could alter endogenous binding and phagocytosis of *S. pneumoniae* by primary murine macrophages, we pre-incubated the bacterium with either folding buffer, BSA or the SRCR construct. It was determined that incubation with the SRCR construct enhanced total cell association by approximately 40% compared with controls, rather than blocking function (Figure 4c).

The SRCR domain of MARCO alters cellular morphology and enhances cellular adhesion

To determine whether the SRCR domain of MARCO contributed to the altered cell morphology that is observed in MARCO-expressing cells, we visualized HEK 293 T cells transfected with myc-MARCO, myc-MARCOII or empty vector control by s.e.m. MARCO-transfected cells produced a large number of thin (< 1 μm), branched,
dendritic-like processes (Figures 5a–d). This phenotype was not observed in MARCOII-transfected cells (Figures 5e–h), indicating that the SRCR domain is required for the production of dendritic-like processes.

To further understand the role of the SRCR domain in cellular adhesion, we quantified cellular adhesion using transiently transfected HEK 293 T cells. HEK 293 T are weakly adherent to tissue culture-treated plastic but were observed to increase in adherence when transfected with MARCO. When adherence was directly quantified by an adhesion assay, MARCO-transfected cells showed a 300% increase in adherence when compared with MARCOII-transfected cells after 45 min of Accutase treatment (Figure 5i). This indicates that MARCO can enhance cellular adhesion via the SRCR domain.

Taken together, these results place an emphasis on the importance of the SRCR domain of MARCO in ligand binding, enhancing pro-inflammatory signaling and modulating cellular adhesion.

**DISCUSSION**

The class A family of scavenger receptors contains five members including SRA, MARCO, SCARA3, SCARA4 and SCARA5. Despite belonging to the same class, the cA-SRs share varying degrees of protein domain homology and, importantly, function.16 The functional heterogeneity of the cA-SRs has made it difficult to assign unifying functions to shared protein domains. This is especially true in the case of the SRCR domain, a domain shared by SRAI, MARCO and SCARA5.

Several MARCO transcript variants have been identified via Aceview human 2010 transcript database (NCBI), yet have never been functionally characterized.12 In this study, we sought to characterize the functional importance of the SRCR domain of MARCO using a naturally occurring transcript variant. We began by confirming that the MARCOII transcript variant existed in human PBMCs (Figure 1b). It has been well demonstrated that MARCO expression is enhanced in macrophages polarized to an M2 phenotype via interleukin-10 treatment.14–16 Therefore, we sought to determine if M1 or M2 polarization of macrophages would alter the expression of the MARCOII transcript. Both M1 polarization via lipopolysaccharide or interferon-γ treatment and M2 polarization via interleukin-10 treatment did not change the expression of the MARCOII transcript (Figure 1d). Interestingly, we do not observe universal or consistent expression between donors implying that there may be additional genetic regulation of MARCOII (Figure 1d). Indeed a number of polymorphisms within introns of MARCO have been associated with increased susceptibility to *Mycobacterium tuberculosis*, but whether this

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**Figure 4** The SRCR domain binds *S. pneumoniae* and enhances NF-κB activity via TLR2/CD14. (a) HEK 293 T cells were transfected with combinations of NF-κB secreted embryonic alkaline phosphatase (SEAP) reporter plasmid, TLR2/CD14, and MARCO or MARCOII. Cells were then stimulated with *S. pneumoniae* for 48 h followed by quantification of NF-κB activity. MARCOII-transfected cells show no significant enhancement of NF-κB activity when compared with MARCO. (b) A soluble SRCR construct binds *S. pneumoniae*. (c) *S. pneumoniae* was pre-incubated with folding buffer, BSA or SRCR construct for 2 h. Bacteria were then incubated with peritoneal macrophages at a multiplicity of infection (MOI) of 25 for 30 min. Percent bacterial association was calculated as the bacteria recovered at 30 min. The relative total cell association was normalized to buffer pre-treated *S. pneumoniae*. Statistical significance was calculated by one-way analysis of variance (ANOVA) with Tukey’s post-hoc test. Error bars indicate mean ± s.e.m. ***P<0.001. All experiments were performed a minimum of three times with a minimum of three technical replicates.
is due to differences in MARCO or MARCOII expression is not known.17,18

Previous work by Brännström et al.5 highlighted the importance of the RxR motif within the SRCR domain of MARCO for ligand binding using artificially truncated or mutated constructs; however, some constructs were not expressed on the cell surface.5 Others have reported similar findings when expressing artificial constructs of SRA.19 We have demonstrated that MARCO and MARCOII are expressed at comparable levels in our transient transfection system by western blotting and that they both expressed on the cell surface by flow cytometry (Figures 2a and b) and IF microscopy (Figures 2c-j). Therefore, differences in function are not due to differences in protein expression.

Identification of the ligand binding site of MARCO has remained especially controversial for multiple reasons. As stated above, Brännström et al.5 have suggested that the unique RxR motif within the SRCR domain of MARCO for ligand binding because of positive charge clustering.20 These findings are consistent with its high affinity for polyanionic ligands. However, SRAI/II and SCARA3 also bind many of the same polyanionic ligands (including modified low-density lipoproteins and bacteria) within the proximal collagenous domain.7,8,11 Thus, both receptors may use regions of positive charge for ligand binding but these regions are localized to different areas of the receptor. To determine the role of the SRCR domain of MARCO in ligand binding, we performed studies using 500 nm Mal-BSA-coated polystyrene microspheres as a ligand for both MARCO and MARCOII. We showed that cells expressing MARCO bound and internalized significantly more microspheres compared with MARCOII (Figures 3i and j). Furthermore, only cells expressing myc-MARCO bound microspheres when analyzed by IF microscopy. Neither myc-MARCOII-expressing nor vector-transfected cells bound a significant number of microspheres (Figures 3a–h).

We hypothesized that MARCOII may act as a dominant negative given that the collagenous domain of cA-SRs is critical for homotrimerization and that both MARCO and MARCOII share identical collagenous domains.11 To test this, we first knocked down ligand binding and internalization of stably expressing MARCO HEK 293 T cells by transfection with MARCOII. We observed a 72% reduction in ligand binding and a 61% reduction in ligand internalization (Figures 3k and l). Conversely, we rescued endogenous function by transfecting stably expressing MARCOII HEK 293 T cells with MARCO and observed a 172% increase in ligand binding and a 218% increase in ligand internalization (Figures 3m and n). Second, we showed that myc-MARCOII can be immunoprecipitated from stably expressing MARCO HEK 293 T cells (Figure 3o). Together, this suggests that MARCO and MARCOII can form heterotrimers and that MARCOII can act as a dominant-negative isoform of the receptor. It has been previously shown that other members of the cA-SRs, namely SRA, can also exist in multiple splice forms, including as a dominant-negative isoform, SRA-III.9 Unlike SRA-III, which is trapped intracellularly, MARCOII can be expressed at the cell surface and likely exerts any dominant-negative function by reducing the
number of SRCR domain-containing moieties within a MARCO/MARCOII heterotrimer. Taken together, these data demonstrate that the SRCR domain of MARCO is the ligand binding site.\textsuperscript{17,18}

MARCO has been shown to have a vital role in the recognition and clearance of bacterial infections in non-opsonic environments, as well as tethering bacterial ligands to other complexes to initiate an inflammatory response.\textsuperscript{13,21} This tethering interaction between MARCO and other pattern recognition receptors (such as TLR2) is a critical step in initiating an innate immune response, as MARCO has never been shown to signal directly. We have previously shown that MARCO is important in the pathway leading to nucleotide oligomerization domain 2 (Nod2) and TLR2-dependent NF-κB activation in response to S. pneumoniae.\textsuperscript{13} Given the important role MARCO has in pro-inflammatory responses to pathogens, we first sought to determine if the SRCR domain of MARCO is required to directly bind S. pneumoniae irrespective of the collagenous domain. We showed by flow cytometry that in addition to Mal-BSA-coated microspheres, the SRCR construct bound S. pneumoniae (Figure 4b). This both confirms that MARCO binds S. pneumoniae and additionally identifies the SRCR domain as the ligand binding site. In fact, the SRCR enhances cell association with S. pneumoniae (Figure 4c). This finding is in agreement with previous findings that the SRCR binds Escherichia coli and enhances phagocytosis of Haemophilus ducreyi by human macrophages.\textsuperscript{15,22} Ligand binding is a critical step not only in phagocytosis, but also for the receptor interactions that are required to signal in response to pathogens. MARCO has been shown to have a vital role in enhancing the NF-κB pro-inflammatory response to M. tuberculosis by tethering the cell wall glycolipid trehalose 6,6'-dimycolate to TLR2/CD14.\textsuperscript{21} We have also shown that MARCO is required for NOD2- and TLR2-mediated responses to unidentified pneumococcal ligands.\textsuperscript{13} To determine if the SRCR domain was required to enhance TLR2-dependent NF-κB activity, we performed an NF-κB reporter assay using cells expressing MARCO or MARCOII. Unsurprisingly, cells transfected with MARCO showed a significant increase in NF-κB activity in response to S. pneumoniae stimulation when compared with cells transfected with TLR2 and CD14 alone. Cells transfected with MARCOII showed no significant change in NF-κB response to S. pneumoniae stimulation when compared with cells transfected with TLR2 and CD14 alone (Figure 4a). These data indicate that the SRCR domain of MARCO is essential to indirectly enhance NF-κB activity via TLR2/CD14.

Apart from enhanced ligand binding, uptake and downstream inflammatory responses, expression of MARCO has also been shown to drastically alter cellular morphology and adhesion.\textsuperscript{23} Pikkarainen et al.\textsuperscript{23} highlighted that MARCO-mediated cellular adhesion is likely modulated by a proximal region (in later publications, identified as the RxR motif) of the SRCR domain. Similar to later studies by the same group, an experimental caveat occurred because of various truncated MARCO constructs showing different levels of expression. In contrast, SRA-mediated adhesion has been shown to be dependent on the collagenous domain, so we sought to characterize the role of the SRCR domain of MARCO in both SR-induced morphological changes and cellular adhesion.\textsuperscript{24-26} Analysis of MARCO-transfected cells by scanning electron microscopy showed that MARCO-transfected cells formed thin (<1 μm), highly branched, dendritic-like processes, as previously observed by Pikkarainen et al.\textsuperscript{23} This morphology was not observed in MARCOII-transfected or vector control cells, which adhered by large, unbranched processes (Figures 5a–h). We next sought to confirm whether the SRCR domain of MARCO directly enhances cellular adhesion of weakly adherent cells. Ojala et al.\textsuperscript{27} previously showed that transfection of HEK 293 cells with SCARA5 greatly enhances cellular adhesion. SCARA5-mediated adhesion is likely not mediated by the SRCR domain, as it does not contain an RxR motif, but this remains to be tested. Preliminary evidence for the role of MARCO in cellular adhesion was observed during passing of stable MARCO-expressing HEK 293 T cells and lifting of transient transfectants. MARCO-expressing cells required significantly more force and repetitions when using forceful pipetting of media as a lifting method. This phenomenon was not observed in vector control or MARCOII-transfected cells. Given that HEK 293 T cells are weakly adherent, we decided to directly quantify if transfection with MARCO enhances cellular adhesion. Over a 45-min time course of Accutase treatment, MARCO-transfected cells remained three times more adherent when compared with MARCOII-transfected cells (Figure 5i). These data suggest that unlike SRA, MARCO relies on the SRCR domain to adhere to surfaces. In addition, our data confirm that the SRCR domain is required to induce MARCO-mediated morphological changes.

These findings show that the SRCR domain of MARCO is critical for receptor function. Given that SRA binds ligands and enhances cellular adhesion independently of the SRCR domain, we can conclude that CA-SR and MARCO are not universally tied to the SRCR domain.

### METHODS

#### Cell lines

HEK 293 T (ATCC #CRL-3216) cells were maintained in complete Dulbecco’s modified Eagle’s medium supplemented with 10% heat inactivated fetal bovine serum, 2 μg/ml streptomycin, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C and 5% CO2. Immunoprecipitation experiments were performed using HEK 293 T cells stably expressing human MARCO. All cell lines were regularly tested for mycoplasma contamination.

#### Plasmids and antibodies

Human MARCO plasmids were provided by Timo Pikkarainen. Human MARCO II cDNA (clone C50DM004HJ08) was subcloned from pCMVSPORT 6 plasmid provided by Wu-Bo Li (GenBank accession number CR603381) into pcDNA 3.1/Hygro\textsuperscript{24} (Invitrogen, Carlsbad, CA, USA) by standard techniques. Human TLR2 and CD14 plasmids were provided by Dr Cynthia Leifer (Cornell University, Ithaca, NY, USA). NF-κB secreted embryonic alkaline phosphatase reporter plasmid was purchased from InvivoGen, San Diego, CA, USA. All plasmids were amplified by chemically competent Escherichia coli DH5α cells (Invitrogen) and purified using a HiPure Plasmid Filter MidiPrep Kit (Invitrogen). Addition of C-terminal myc tags to MARCO and MARCOII was performed by PCR amplification with primers that contained the myc sequence and restriction enzyme sites to facilitate cloning.

Primary antibodies that were used included; monoclonal mouse anti-beta actin (Sigma-Aldrich, Oakville, Ontario, Canada), monoclonal mouse anti-Myc (9E10) and a rabbit polyclonal anti-MARCO (cytoplasmic domain), as in Eloma et al.\textsuperscript{25} MARCO (SRCR) and MARCOII-specific rabbit polyclonal immunoglobulin G (IgG) was produced by immunizing a New Zealand White rabbit with a hapten consisting of the 21 C-terminal amino acids of MARCO or MARCOII with adventitious K and Y residues for KLH coupling. Serum was collected and purified using a HiPure Plasmid Filter MidiPrep Kit (Invitrogen). Addition of C-terminal myc tags to MARCO and MARCOII was performed by PCR amplification with primers that contained the myc sequence and restriction enzyme sites to facilitate cloning.
Human macrophage culture and transcript detection

PBMCs were collected from donors who provided informed written consent. All studies were approved by the Hamilton Integrated Research Ethics Board. PBMCs were isolated from buffy-coat preparations by Ficoll density gradient centrifugation and differentiated for 7 days in X-VIVO 10 culture media (Lonza, Basel, Switzerland) supplemented with 5% human AB serum (Lonza) and 20 ng ml⁻¹ granulocyte-macrophage colony-stimulating factor.

PBMCs were stimulated for 48 h with either PBS (unstimulated, US), lipopolysaccharide (100 ng ml⁻¹), interleukin-10 (20 ng ml⁻¹) or interferon-γ (20 ng ml⁻¹). Following stimulation, cells were lysed and RNA was isolated using a GENEzol TriRNA Pure Kit (Froggabio, North York, ON, Canada) following the manufacturer’s protocol. cDNA was synthesized from 2 μg PBMC RNA using Moloney murine leukemia virus reverse transcriptase (New England Biolabs, Ipswitch, MA, USA) following the manufacturer’s protocol. Semi-quantitative PCR was then performed for 30 cycles using primers surrounding exons 14/17 of the human MARCO mRNA transcript or glyceraldehyde-3-phosphate dehydrogenase as a housekeeping control transcript (Table 1). PCR products were run on a 2% agarose gel, stained with ethidium bromide and imaged on an Alphalmager Imaging System (Alpha Innotech, San Leandro, CA, USA). To confirm each transcript identity, bands were excised and purified using a GenElute Gel extraction kit (Sigma-Aldrich). Sequencing was performed at the McMaster University MOBIX facility.

Production of recombinant SRCR domain

The SRCR region of MARCO (residues 400–520) was subcloned into a modified pET15b bacterial expression plasmid including a 6× His tag followed by a tobacco etch virus protease cleavage site. The integrity of the resulting His₅-SRCR construct was confirmed by DNA sequencing (MOBIX, McMaster University). *Escherichia coli* Rosetta-Gami2 cells (Novagen, San Diego, CA, USA) were transformed and grown in Luria–Bertani media to an OD₆₀₀ ~0.7.

Bacterial cultures were grown in complete Dulbecco’s modified Eagle’s medium –mercaptoethanol. The bacterial cultures were harvested by centrifugation at 3500 g for 15 min. Cell pellets were resuspended in lysis buffer (20 μM Tris pH 8, 500 mM NaCl, 2.8 mM β-mercaptoethanol, 5% v/v glycerol) supplemented with protease inhibitors (phenylmethylsulfonyl fluoride, leupeptin, aprotinin and pepstatin A) and lysed by sonication. As the SRCR domain was found in the supernatant monolayer was observed. In order to measure the strength of adhesion, cells were treated with 1 ml Accutase (BD Biosciences, Mississauga, Canada). To determine green fluorescent protein expression. The cultures were then read on a SpectrMax 384 Plus spectrophotometer (Molecular Devices) at 655 nm absorbance to determine secreted embryonic alkaline phosphatase expression (NF-κB activity). NF-κB activity (Abs₆₅₅nm) was normalized by dividing secreted embryonic alkaline phosphatase activity by green fluorescent protein expression.

**Microsphere binding assays**

HEK 293 T cells were seeded in complete Dulbecco’s modified Eagle’s medium supplemented with 10% heat inactivated fetal bovine serum, 2 mM β-mercaptoethanol, 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin at 1×10⁵ cells per well into six-well plates. Plates were transfected each with pcDNA3.1/Hygro (+), MARCO or MARCOII plasmids at 2 μg per well using polyethyleneimine (Sigma-Aldrich). For knockdown and rescue-of-function experiments, stably expressing MARCO or MARCOII HEK 293 T cells were transiently transfected as above with either 2 μg pcDNA3.1/Hygro (+), 0.5 μg MARCOI+1.5 μg pcDNA3.1/Hygro (+) or 0.5 μg MARCOI+1.5 μg pcDNA3.1/Hygro (+).

At 48 h post transfection, cells were lifted, pooled and centrifuged at 400 g for 10 min. Media were removed and cells were resuspended in Opti-Mem (Invitrogen). Cell numbers were normalized in 1 ml aliquots of each transfectant and aliquoted into replicates with conditions of ± microspheres and 4 °C or 37 °C. In all, 0.5 μm polystyrene yellow fluorescent microspheres (Polysciences, Warrington, PA, USA) passively coated with Mal-BSA were added to the cells at approximately 320 microspheres per cell. The tubes were then incubated with gentle agitation at 4 °C and 37 °C for 1.5 h. Following incubation, the cells were centrifuged for 10 min at 500 g. The media were removed from each tube and cells were washed 2x with 1 ml PBS to remove all unbound microspheres. Following washes, the cells were centrifuged at 500 g for 10 min at room temperature and resuspended in 200 μl PBS. Samples were added to a black 96-well plate and fluorescence was measured on a Spectrax M3 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at Ex₄₄₁nm/Em₄₈₆nm. Given that ligand binding, but not phagocytosis occurs at 4 °C, we calculated microsphere internalization by subtracting the relative fluorescence of cells incubated at 4 °C (bound microspheres) from cells incubated at 37 °C (total microsphere association; bound and internalized microspheres). We performed microsphere binding and internalization experiments using chlorpromazine, which inhibits chlorinated-mediated internalization to demonstrate that microsphere internalization occurred only at 37 °C (Supplementary Figure S2).

**Table 1 Primers sequences used for semiquantitative PCR and transcript sequencing**

| Gene   | Forward (5’)    | Reverse (3’)    |
|--------|-----------------|-----------------|
| hMARCO | AGGTGTGAAAGGGAACAGG | GTGCCAGCTCCACAGGGTACT |
| hGAPDH | GAGTCAAGGTGGTTTGCAGT | TTGATTTGGAAGGATCTCG |

**NF-κB reporter assays**

HEK 293 T cells were seeded in complete Dulbecco’s modified Eagle’s medium at 10⁵ cells per well in 96-well plates. Cells were transfected with combinations of plasmids as in Bowdish et al.²¹ *S. pneumoniae* serotype 23 F, clinical isolate P1121 was grown and prepared as in Dorrington et al.¹³ and McCool et al.⁰² Cells were stimulated 48 h post transfection using HEK Blue detection media (InvivoGen) supplemented with 1 μg ml⁻¹ of TLR2 agonist Pam3Csk4 (InvivoGen) as a positive control, or heat-killed, lysozyme-digested *S. pneumoniae* (multiplicity of infection = 50). NF-κB activation was measured for Pam3Csk4-stimulated cultures after 24 h and *S. pneumoniae*-stimulated cultures after 48 h. Cells were first analyzed on a Typhoon Trio variable mode imager and quantified using ImageQuant software (ImageMaster, Ann Arbor, MI, USA) to determine green fluorescent protein expression. The cultures were then read on a SpectrMax 384 Plus spectrophotometer (Molecular Devices) at 655 nm absorbance to determine secreted embryonic alkaline phosphatase expression (NF-κB activity). NF-κB activity (Abs₆₅₅nm) was normalized by dividing secreted embryonic alkaline phosphatase activity by green fluorescent protein expression.

**Cellular adhesion assays**

HEK 293 T cells were seeded in complete Dulbecco’s modified Eagle’s medium at 1×10⁵ cells per well into six-well plates. Cells were transfected with plasmids expressing MARCO, MARCOII or with an empty vector control. At 48 h post transfection, a confluent monolayer was observed. In order to measure the strength of adhesion, cells were treated with 1 ml Accutase (BD Biosciences, Mississauga, Ontario, Canada), an enzymatic cocktail containing EDTA, which eliminates integrin-mediated, but not MARCO-mediated adhesion. After 0 h, 15, 30 and 45 min of accutase treatment, cells were washed and adherent cells were quantitated. Each well was stained with 1 ml crystal violet for 2 min and washed three times with water to remove excess dye. Plates were dried overnight at room temperature. Following drying, 1 ml 0.2% sodium dodecyl sulfate solution was added to each well. Relative amount of cell adhesion was quantitated by measuring the Abs₅₅₀nm on a Nanovue spectrophotometer (GE Healthcare).
IF microscopy

IF microscopy was performed using cells adherent on poly t-lysine-coated 24-well glass cover slips. Samples were fixed in 2% paraformaldehyde (pH 7.4) at 37 °C for 10 min followed by three washes with PBS for 10 min. Slides were blocked with 5% BSA for 1 h at room temperature and stained overnight with mouse anti-Myc at 4 °C. Samples were then washed three times with PBS, and stained with Alexa Fluor 488 or 633 goat anti-mouse IgG (Invitrogen) or Texas Red Phalloidin (Invitrogen) for 30 min at room temperature. Samples were washed a final three times with PBS for 10 min. Slides were mounted with ProLong Gold (Invitrogen) and were imaged on a Leica DM IRE2 inverted fluorescence microscope (Leica, Wetzlar, Germany) and adjusted for brightness and contrast using OpenLab 5.5.0 and ImageJ (NIH, Bethesda, MD, USA). All adjustments were applied equally to all images.

Electron microscopy

Samples were immersed overnight in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). The samples were rinsed twice in buffer solution and post-fixed for 1 h in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. After the second fixation step, the samples were dehydrated through a graded ethanol series (50, 70, 70, 95, 95, 100, 100%) and then dried in a critical point dryer. After drying, the samples were mounted onto scanning electron microscopy stubs. The stubs were sputter coated with gold and viewed with a Tescan Vega II LSU scanning electron microscope (Tescan, Brno, Czech Republic).

Flow cytometry and SRCR construct experiments

To evaluate surface expression of MARCO and MARCOII, cells were stained with 9E10 mouse anti-myc antibodies in 5% BSA for 1 h at room temperature followed by two washes with PBS. Secondary staining was performed using Alexa Fluor 633 goat anti-mouse IgG (Invitrogen) antibodies in 5% BSA for 30 min at room temperature in the dark. Following staining, cells were washed twice with PBS, filtered and assayed with a BD FACsCanto II flow cytometer (BD Biosciences).

S. pneumoniae was prepared as above. In all, 5x10^5 bacteria or BSA/Mal-BSA-coated microspheres were incubated with 40 μg SREC construct in folding buffer at room temperature for 2 h. Cells were washed with PBS and stained as above using a rabbit polyclonal anti-SREC antibody in 0.1% BSA for 1 h at room temperature. Following staining, cells were washed twice with PBS, filtered and assayed with a BD FACsCanto II flow cytometer.

Data were gathered using FACS Diva software (BD Biosciences) and analyzed using Flowjo version 7.6.2 software (TreeStar, Ashland, OR, USA).

Quantification of bacterial association was performed by incubating 5x10^7 S. pneumoniae with either folding buffer alone, 40 μg BSA or 40 μg SREC construct in folding buffer at 4 °C for 2 h. Cells were washed twice in Hanks balanced salt solution. Biogel elicted peritoneal macrophages were collected from wild-type C57Bl/6 mice as in Dorrington et al.13 Approximately 2x10^6 macrophages were infected at a multiplicity of infection of 25 for 30 min. Macrophages were washed once with PBS to remove non-associated bacteria followed by lysis in sterile water. Serial dilutions were performed in water and plated on sheep’s blood agar supplemented with 10 μg ml^-1 neomycin as in Dorrington et al.13 Colonies were counted after overnight incubation at 37 °C.

Statistics

Statistical analyses were performed using GraphPad Prism 5.01 (Graphpad Software, San Diego, CA, USA). Results were considered statistically significant if P<0.05.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Author contributions: KEN designed, performed and analyzed the experiments and wrote the manuscript. AH created and purified the construct used in Figure 4b and Figure S3. SJH performed and analyzed the experiments shown in Figure 4a. MGD performed and analyzed the experiments shown in Figure 4b and Figure S3. CDW designed the experiments shown in Figure 4b and Figure S3. ZT designed and performed the experiments in Figure S2. PP and WD reviewed the procedures used to generate polyclonal rabbit antibodies. AG and KS edited the manuscript and provided experimental critique. DMEB designed the experiments and planned and edited the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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1. Canton J, Neculai D, Grinstein S. Scavenger receptors in homeostasis and immunity. Nat Rev Immunol 2013; 13: 621–634.
2. Geaves DR, Gordon S. The macrophage scavenger receptor at 30 years of age: current knowledge and future challenges. J Lipid Res 2009; 50 (Suppl): S282–S286.
3. Krala G, van der Laan LJ, Elomaa O, Tryggvason K. The macrophage receptor MARCO. Microbes Infect 2000; 2: 313–316.
4. Martinez VG, Moestrup SK, Holmskov U, Mollenhauer J, Lorzo F. The conserved scavenger receptor cysteine-rich superfamily in therapy and diagnosis. Pharmacol Rev 2011; 63: 967–1000.
5. Brännström A, Sankala M, Tryggvason K, Pikkarainen T. Arginine residues in domain V and low affinity ligand binding properties of murine type I and type II macrophage scavenger receptor MARCO. Biochem Biophys Res Commun 2002; 290: 1462–1469.
6. Whelan FJ, Meehan CJ, Goldberg GB, McConkey BJ, Bowdish DME. The evolution of the class a scavenger receptors. BMC Biol 2012; 13: 227.
7. Rohrer L, Freeman M, Kodama T, Penman M, Krieger M. Coiled-coil fibrous domains mediate ligand binding by macrophage scavenger receptor type II. Nature 1990; 343: 570–572.
8. Mori K, Ohtani K, Jang S, Kim Y, Huang I, Roy N et al. Scavenger receptor CL-P1 mainly utilizes a collagen-like domain to uptake microbes and modified LDL. Biochim Biophys Acta 2014; 1840: 1–12.
9. Gough PJ, Geaves DR, Gordon S. A naturally occurring isomorph of the human macrophage scavenger receptor (SR-A) gene generated by alternative splicing blocks modified LDL uptake. J Lipid Res 1998; 39: 531–543.
10. Jozefowski S, Arendoudani M, Sulahian T, Kobikli L. Disparate regulation and function of the class A scavenger receptors SR-A/II and MARCO. J Immunol 2005; 175: 799–804.
11. Ashkenazi J, Penman M, Vialle E, Acton S, Freeman M, Krieger M. Structures and high and low affinity ligand binding properties of murine type I and type II macrophage scavenger receptors. J Lipid Res 1993; 34: 983–1000.
12. Thierry-Mieg D, Thierry-Mieg J. AceView: a comprehensive cDNA-supported gene and transcripts annotation. Genome Biol 2006; 7 (Suppl 1): S12.1–14.
13. Dorrington MG, Roche AM, Chauvin SE, Tu Z, Moshans KL, Weiser JO et al. MARCO is required for LR2- and Nod2-mediated responses to Streptococcus pneumoniae and clearance of pneumococcal colonization in the murine nasopharyx. J Immunol 2013; 190: 250–258.
14. Montoya D, Cruz D, Teles RMB, Lee DJ, Oh Choa MT, Kruutz SK et al. Divergence of macrophage phagocytic and antimicrobial programs in leprosy. Cell Host Microbe 2009; 6: 343–353.
15. Li W, Katz BP, Spinola SM. Haemophilus ducreyi-induced interleukin-10 promotes a mixed M1 and M2 activation program in human macrophages. Infect Immun 2010; 80: 4426–4434.
16. Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: an immunologic functional perspective. Annu Rev Immunol 2009; 27: 451–483.
17. Bowdish DM, Sakamoto K, Lack NA, Hill PC, Sirugo G, Newport MJ et al. Genetic variants of MARCO are associated with susceptibility to pulmonary tuberculosis in a Gambian population. BMC Med Genet 2013; 14: 47.
18. Ma M-J, Wang H-B, Li H, Yang J-H, Yan Y, Xie L-P et al. Genetic variants in MARCO are associated with the susceptibility to pulmonary tuberculosis in Chinese Han population. PLoS ONE 2011; 6: e24096.
19. Huang F-L, Shao Y-J, Hou S-J, Yang C-N, Chen Y-J, Lin C-H et al. Cysteine-rich domain of scavenger receptor AI modifies the efficacy of surface targeting and mediates oligomeric Abeta internalization. J Biomed Sci 2013; 20: 54.
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