Characterization of Recombinant Soluble Macrophage Scavenger Receptor MARCO*

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MARCO is a type II transmembrane protein of the class A scavenger receptor family. It has a short N-terminal cytoplasmic domain, a transmembrane domain, and a large extracellular part composed of a 75-residue long spacer domain, a 270-residue collagenous domain, and a 99-residue long scavenger receptor cysteine-rich (SRCR) domain. Previous studies have indicated a role for this receptor in anti-microbial host defense functions. In this work we have produced the extracellular part of MARCO as a recombinant protein, and analyzed its binding properties. The production of this protein, soluble MARCO (sMARCO), has made it possible for the first time to study MARCO and its binding properties in a cell-free system. Using circular dichroism analyses, a protease-sensitive assay, and rotary shadowing electron microscopy, sMARCO was shown to have a triple-helical collagenous structure. Rotary shadowing also demonstrated that the molecules often associate with each other via the globes. sMARCO was found to bind avidly both heat-killed and living bacteria. Lipopolysaccharide, an important component of the outer membrane of Gram-negative bacteria, was shown to be a ligand of MARCO. Studies with different bacterial strains indicated that the O-side chain of lipopolysaccharide is not needed for the bacterial recognition. Finally, the C-terminal SRCR domain was also produced as a recombinant protein, and its bacteria-binding capability was studied. Although the transfection experiments with transmembrane MARCO variants have indicated a crucial role for this domain in bacterial binding, the monomeric domain exhibited low, barely detectable bacteria-binding activity. Thus, it is possible that cooperation between the SRCR domain and the collagenous domain is needed for high-affinity bacterial binding, or that the SRCR domain has to be in a trimeric form to effectively bind to bacteria.

Innate immunity, the first line of defense against infectious microorganisms, appeared early in evolution. Innate immunity relies on specialized cells such as macrophages that are the first to encounter pathogens during infection (1, 2). Macrophages have a major role in host defense, but they also function in normal physiological processes, such as in the maintenance of tissue homeostasis. Macrophages are found in many tissues, but particularly in those that function in the filtration of blood or lymph fluids, such as liver, spleen, lung, and lymph nodes. They recognize, internalize, and destroy harmful endogenous and foreign substances, thus functioning as scavengers. Macrophages have been shown to be able to bind pathogens directly, or they recognize them as foreign after being coated with antibodies or complement (3). Macrophages contain a set of non-clonal receptors that can directly recognize pathogen-associated common structures. These receptors, known as pattern-recognition receptors, include the mannose receptor, CD14, Toll-like receptors, and scavenger receptors (SRs) (4). A wide variety of pathogen-associated structures are recognized by the pattern-recognition receptors. These include mannans and zymosan in the yeast cell wall, and various bacterial cell-wall components, such as lipopolysaccharide (LPS), lipoproteins, lipoteichoic acid, and peptidoglycans (4). Many of these components are able to stimulate the innate immune system. The best known example is LPS, the major outer membrane component of all Gram-negative bacteria. LPS is composed of a lipophilic component, lipid A, and a poly- or oligosaccharide portion covalently linked to this membrane anchor domain (5). LPS is a very potent activator of innate immune responses, and thus it is the major inducer of septic shock during infections caused by Gram-negative bacteria.

Of the several classes of SRs, primarily the class A SRs are considered to be of importance for the anti-microbial host defense. This class contains three members, SR-A (scavenger receptor A), MARCO (macrophage receptor with collagenous domain), and a recently identified protein SRCL (scavenger receptor with C-type lectin) (6). SR-A, which is expressed in most macrophage populations, is a trimeric membrane protein containing an N-terminal intracellular domain, a transmembrane domain, and an extracellular portion composed of a short spacer domain, an α-helical coiled-coil domain, a triple-helical collagenous domain, and a C-terminal cysteine-rich domain (7, 8). As a result of alternative splicing of the primary transcript, SR-A exists in two forms, SR-AI and SR-AII, of which the latter lacks the SRCR domain (9). Both receptors bind a large number of polyanionic molecules, including the prototypic SR ligand, modified low density lipoprotein, as well as LPS and lipotei-
choic acid. Studies with SR-A-deficient mice have indicated a role for SR-A in the formation of atherosclerotic lesions (10). In addition, the SR-A-deficient mice were found to have increased susceptibility to infections caused by *Listeria* monocytogenes and *Staphylococcus aureus* (10, 11). This finding, together with the data that SR-A binds bacterial cell-wall components, clearly indicates a role for SR-A in the innate immune system.

MARCO differs structurally from SR-A in that it lacks the α-helical coiled-coil domain, but has, instead, a long collagenous domain (12). Thus, MARCO has a short N-terminal intracellular domain, a transmembrane domain, and a large extracellular part comprised of a spacer domain, the long collagenous domain, and an SRCR domain (domain V). The SRCR domain contains six cysteine residues that form three intrachain disulfide bridges (13). The amino acid sequence of this domain has 46% identity with that of the SRCR domain of the Mac-2-binding protein, whose crystal structure has been solved (14). In normal mice, MARCO is expressed only on marginal zone macrophages of the spleen and on macrophages of the medullary cord in lymph nodes (12), i.e. on cells that are strategically positioned to capture pathogens from the blood and lymph nodes. In line with the concept that MARCO plays a role in antimicrobial host defense functions, its expression is up-regulated in bacterial infections in macrophages of most tissues (13, 15–17). Moreover, cells transfected with the MARCO expression plasmid avidly bind both Gram-negative and Gram-positive bacteria, but not yeast (12, 13). Studies with MARCO variants have shown that the SRCR domain, domain V, is the predominant bacteria-binding domain of MARCO (13, 18). Because the collagenous domain has been shown to be the ligand-binding domain of SR-A, MARCO and SR-A have different ligand-binding properties. No function has yet been found for the SRCR domain of SR-A.

Even if the MARCO-expressing transfected cells bind bacteria, it is possible that the cell-bacteria interactions are not directly mediated by MARCO, but result from some secondary changes in the transfected cells. This possibility has to be taken into consideration, especially because MARCO expression induces dramatic phenotypic changes in the transfected cells (19). Thus, we unequivocally demonstrate that MARCO binds bacteria, one needs to assay the bacteria-binding activity of isolated MARCO molecules. This is one reason why we have undertaken the approach of producing “soluble MARCO” (called hereafter sMARCO), i.e. a recombinant MARCO protein composed of the entire extracellular part, but lacking the N-terminal cytoplasmic and transmembrane domains. The His-tagged sMARCO was purified to homogeneity from 293/EBNA cell-conditioned medium, and its structure was studied. sMARCO was found to form stable elongated triple-helical molecules. Functional analyses showed that sMARCO indeed binds bacteria with high affinity. Additional studies indicated that MARCO is able to bind LPS. In light of this finding, it will be of interest to compare the responses of wild-type and MARCO-deficient mice to the LPS-induced endotoxic shock. Finally, we have also produced and purified recombinant domain V (called hereafter rev), and studied whether a surface coated with this protein supports binding of bacteria. This study indicated that compared with sMARCO, the monomeric rev protein has low, barely detectable bacteria-binding activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Mammalian episomal expression vectors pCEP-Pu and pCEP-Pu/AC7 were kind gifts from Drs. T. Sasaki and R. Timpl (Max Planck Institute for Biochemistry, Martinsried, Germany). The rat anti-mouse MARCO monoclonal antibody ED31 was kindly provided by Dr. G. Kraal (Free University, Amsterdam, The Netherlands). The wild-type *Escherichia coli* strain R12 was from ATCC. *E. coli* strain HB101 and *Salmonella typhimurium* gale mutant strain LB5010 were generously provided by Dr. Mikael Rhen (Karolinska Institutet, Stockholm). Mouse L929 fibroblasts were from ATCC. Dulbecco’s modified Eagle’s medium (DMEM), DMEM/F-12, fetal calf serum, the human embryonic kidney epithelial 293/EBNA cell line, pSec-Tag2 vector, and G418 were obtained from Invitrogen. Antibodies were purchased from New England Biolabs. Ascorbic acid, heparin, polyinosinic acid (poly(I)), puromycin, tunicamycin, and trypsin were from Sigma. Unlabeled and FITC-labeled LPS (from *E. coli* serotype 0111:B4) were also from Sigma. Unlabeled LPS, which was used in the binding assay with sMARCO, had been phenol-extracted and purified by ion-exchange chromatography (L-3024). Secondary antibodies were purchased from Dako or Molecular Probes. Ni-NTA-agarose was from Qiagen. FITC was obtained from Fluka. SYPRO™ Ruby protein gel stain and Pro-Q™ Emerald 300 lipopolysaccharide gel stain kit were purchased from Molecular Probes.

**Expression Constructs**—All DNA manipulations were carried out using established molecular biological methods. To generate the plasmid directing the expression of sMARCO, the region encoding the cytoplasmic and transmembrane domains in the full-length mouse MARCO cDNA was replaced by a fragment encoding the mouse immunoglobulin κ (Igκ) chain leader sequence followed by a 6-residue polyhistidine tag (Fig. 1A). This plasmid was constructed in the following way. First, a PCR fragment encoding the mouse Igκ chain leader sequence (Igκ-chain leader sequence) was inserted into the vector pCEP-Pu (20). This fragment was amplified with the primers 5′-ATGGAGACAGACACACTC-3′ and 5′-CCCCAGCTTGACGGC- GCGCGCT (HindIII-recognition site underlined) using the pSec-Tag2 vector as a template. The fragment was cloned into the *Kpn*I-*Klenow-HindIII*-treated pCEP-Pu vector. Next, a PCR fragment encoding for the entire extracellular part of MARCO (nucleotides 223–1557) was inserted into the HindIII and NotI sites of this vector. The 1.5-kb MARCO fragment was generated using primers 5′-CCCAAGCTTCAGGACAGCTCCGATG-3′ and 5′-ATAGTTTGACCGCGCCTAGGAGCATTCCACACC-3′ (HindIII and NotI recognition sites underlined). Finally, a fragment encoding the histidine tag was inserted into the HindIII site. The fragment was generated by annealing two complementary oligonucleotides with the sequences 5′-ACGTTCGATCCATCG- AC-3′ and 5′-AGTTGATCGTTGATC-3′. Annealing of the oligonucleotides generated HindIII-compatible overlaps, and the fragment could thus be inserted into the expression construct without further manipulations. The sequence of the expression cassette was verified by DNA sequencing.

To generate the domain V-expressing construct, a fragment encoding the C-terminal portion of the α3(VI)-collagen was excised from the vector pCEP-Pu/AC7 with *NheI* and *BamHI*, and was replaced by a 300-bp *NheI*/*BglII* fragment encoding mouse MARCO domain V. This fragment was amplified using pSG5 full-length mouse MARCO as a template. In this plasmid, a BglII site was immediately following the translation stop codon. The sense primer (5′-GTCAAGCTGGCCAAAAGCCTCGGGGATG-3′) contained an *NheI* recognition site (underlined). The antisense primer (5′-GGCTCCACACTAGGAATGCTGCGTG-3′) was derived from the vector sequence 3′ of the BglII site. The resulting construct encoded a protein with the BM-40 signal sequence followed by the first four N-terminal residues of BM-40 (APLA) and mouse MARCO domain V starting from residue Gin18. The authenticity of the construct was confirmed by sequencing.

**Generation of 293/EBNA Cell Clones Producing the Recombinant Proteins**—The recombinant proteins were expressed using the 293/EBNA cell expression system. The human embryonic kidney epithelial 293/EBNA cells were cultured according to the manufacturer’s instructions. This cell line constitutively expresses the EBNA-1 protein of Epstein-Barr virus, thus allowing episomal replication of a vector containing the Epstein-Barr virus origin of replication. The cells were grown in DMEM containing 10% fetal calf serum and 250 μg/ml neomycin analog G418. Cells in 10-cm culture dishes were transfected using the calcium-phosphate method with 20 μg of the expression vector. The transfected cells were selected with 2–3 μg/ml puromycin. Puromycin-resistant clones were tested for the recombinant protein expression by growing the cells in serum-free medium for 24 h. When analyzing the sMARCO production, ascorbic acid (100 μg/ml) was added in the medium. The production of sMARCO was confirmed by dot-blot analysis with the rat anti-mouse MARCO mAb ED31 (15). It has been shown earlier that the epitope for ED31 resides in domain V (16, 18).

**Production and Purification of Soluble MARCO**—A clone expressing sMARCO was grown to confluency in DMEM/fetal calf serum, washed twice with phosphate-buffered saline to remove serum proteins, and
switched to serum-free DMEM/F-12 containing 250 μg/ml G418, 1 μg/ml puromycin, and 100 μg/ml ascorbic acid. Freshly made ascorbic acid was added every 24 h. The medium was collected and exchanged with fresh medium every 72 h over a 15-day period. The collected medium was centrifuged to remove cellular debris and frozen for storage. The purification of sMARCO by recV, the serum-free conditioned medium was produced as described for some of the assays, sMARCO-coated surfaces were incubated with polyanions polyI(1) or heparin before adding the bacteria. PolyI(1) was used at a concentration of 300 μg/ml. Heparin was used at the same concentration or at 1 mg/ml. Polyanions were also present in the bacteria-containing solution. Following incubation with the bacteria, the coverslips were washed three times with buffer B. Bacterial binding was analyzed by fluorescent microscopy.

Production and Purification of Domain V—For the purification of recV, the serum-free conditioned medium was produced as described for sMARCO, except that the cells were grown in serum-free DMEM/F-12 medium for 9 days (medium changed every 72 h). EDTA (5 mM) and phenylmethylsulfonyl fluoride (1 mM) were added to the collected conditioned medium. The medium was dialyzed against 10 mM NaH_{2}PO_{4}, pH 4.0, to remove denaturating compounds like 0.1% SDS and 10% glycerol. The dialyzed medium was centrifuged to remove cellular debris and frozen for storage.

The enzyme was omitted. The samples were analyzed by SDS-PAGE and silver staining under reducing conditions. When analyzed by Western blotting, samples were not reduced because the mAb ED31 does not recognize the reduced domain V. The protein was stored in small aliquots at −80 °C.

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mouse immunoglobulin MARCO and sMARCO. The secreted, soluble form of MARCO was generated by replacing the intracellular and transmembrane domains with the

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ATION. To study whether sMARCO contains

recV, purified sMARCO with anti-mouse MARCO mAb. Electrophoresis was performed under nonreducing conditions. A fraction of the sMARCO molecules have associated covalently into a trimeric molecule. D, the purified recombinant domain V was analyzed by 4–20% SDS-PAGE and silver staining under reducing (lane 1) and nonreducing conditions (lane 2). Molecular mass standards are expressed in kilodaltons.

mass of sMARCO deduced from the cDNA sequence is only 47.1 kDa, but it is typical for collagenous polypeptides that they migrate slower than expected on SDS-PAGE (see, e.g., Refs. 24 and 25). Yet, at least a part of this size discrepancy could be because of post-translational modifications, such as glycosylation. To study whether sMARCO contains N-linked oligosaccharides, the purified protein was subjected to N-glycosidase F digestion. sMARCO contains two potential N-glycosylation sites, both of which reside in the spacer domain. Treatment of the denatured form of purified sMARCO by N-glycosidase F resulted in two bands with apparent molecular masses of 66 and 62 kDa (Fig. 1B, lane 2). Assuming that the molecular mass of an N-linked oligosaccharide is ~3 kDa (26), the 72-kDa form of sMARCO appears to contain two N-linked oligosaccharides, whereas only one of the two potential N-glycosylation sites is occupied by an oligosaccharide in the 65-kDa form. The remaining size difference (4 kDa) between the two sMARCO forms could be because of another type of post-translational modification than N-glycosylation. Alternatively, it is because of proteolytic processing. We also attempted to produce the form of sMARCO lacking the N-linked carbohydrates by culturing the sMARCO-expressing cells in the presence of tunicamycin, an inhibitor of N-glycosylation. However, when analyzing a sample corresponding to 10 ml of the conditioned medium from the cells grown in the presence of tunicamycin for 3 days, no sMARCO could be visualized on a silver-stained gel (not shown). At the same time, a strong signal was obtained from the control sample, i.e., the conditioned medium from the cells grown in the absence of tunicamycin.

Analysis of purified sMARCO under nonreducing conditions indicated two bands, the ~70-kDa band and a slowly migrating band with an apparent molecular mass of ~220 kDa, the expected size of a trimer (Fig. 1C). Thus, a fraction of the sMARCO molecules synthesized in the 293/EBNA cells appears to form disulfide bond-stabilized trimeric molecules. There are two cysteine residues in the spacer domain (12), and it is apparent that these residues form interchain disulfide bonds after triple-helix formation.

Production and Purification of Domain V—Domain V was directed into the secretory pathway using the BM-40 signal sequence. The protein was purified from the serum-free conditioned medium to homogeneity using cation exchange and hydrophobic interaction chromatography. The yield was 2–5 mg/liter medium. N-terminal sequencing gave the peptide sequence APLAQ, which is in perfect agreement with the expected sequence (see “Experimental Procedures”). When analyzed on SDS-PAGE under reducing conditions, recV migrated as a 14–15-kDa band (Fig. 1D, lane 1), considerably slower than expected from the calculated molecular mass (11.3 kDa). However, the mass determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (mlz 11,321 ± 2 for the protonated molecule) agrees well with the predicted size. The nonreduced protein migrated on SDS-PAGE somewhat faster than the reduced protein (Fig. 1D, lane 2), in keeping with the fact that the six cysteines of the domain form three intrachain disulfide bonds (13).

Conformation and Stability of sMARCO—As mentioned above, analysis of purified sMARCO on SDS-PAGE under nonreducing conditions suggested that at least a fraction of the sMARCO molecules have assembled into a triple helix. To get more information about the structure of sMARCO, the purified protein was subjected for circular dichroism analysis. At 20 °C, purified sMARCO produced a spectrum typical for triple-helical collagens, with a negative minimum peak at 198 nm and a positive maximum at around 220 nm (27) (Fig. 2A). The thermal stability was assayed by monitoring the CD signal at 220 nm as a function of temperature. The melting curve indicated a Tm of 44 °C, and further conformational changes at higher temperatures (Fig. 2B).
The folding of sMARCO was also probed using limited trypsin digestion. This assay can be used to study the folding of sMARCO, because collagenous triple helices are resistant to trypsin. The results demonstrated that a major fraction of purified sMARCO has folded into a triple helix. Thus, whereas the 72-kDa form is, by far, the predominant form of sMARCO in the sample incubated without trypsin (Fig. 3, lane 1), trypsin digestion greatly increases the amount of the 65-kDa form, so that after a 2-h digestion there was more of this form than of the 72-kDa form (Fig. 3, lane 2).

Rotary Shadowing—Having shown that sMARCO forms collagenous molecules, the protein was subjected to rotary shadowing electron microscopy analysis to obtain information about the shape and dimensions of the molecule. This analysis revealed dumbbell-shaped particles with globular domains at both ends interlinked by a rod-like domain of 82.7 - 2 nm average length (Fig. 4, A and B). For the evaluation of the average length of the rods from size distributions of more than 100 particles (Fig. 5), a small fraction (<5%) of particles shorter than 60 nm were ignored and considered to be fragments or impurities. The length of the rod-like domain agrees with the calculated length of the triple-helical collagen domain. This length of 80 nm is obtained by multiplication of 270 residues in a strand with the translation of 0.894 nm per tripeptide unit (28). The globular domains correspond to the spacer and the SRCR domains, but it is not possible to distinguish these domains morphologically.

About 50% of all molecules are in the monomeric form and 50% are associated by the globes (Fig. 4, A and C). Association by globes leads to linear dimers and some linear trimers (10%...
of all species involving 25% of the molecules). There are also many star-like complexes (Fig. 4C) in which 3–7 molecules are connected by terminal globes (11% of all species involving 55% molecules).

**Ligand Binding Studies**—Several assays were used to test the ligand-binding properties of sMARCO. First, we wanted to examine whether it can bind bacteria. As already mentioned above, transfected cells expressing the full-length MARCO bind bacteria avidly. However, extensive morphological changes occur in these cells, and it cannot be excluded that bacterial binding is not directly mediated by MARCO. To address this question, we assayed the binding of heat-killed FITC-labeled *E. coli* to a sMARCO-coated glass coverslip (Fig. 6A). Binding of bacteria to coverslips coated with rNephrin (Fig. 6C) or left uncoated (Fig. 6D) was considered as background binding. In this assay, we also assessed the bacteria binding ability of recV (Fig. 6B). This was of interest to study because our binding studies with transmembrane MARCO variants have indicated a crucial role for domain V in the bacteria-binding activity of MARCO (13, 18). As shown in Fig. 6A, sMARCO binds bacteria very strongly. In comparison, recV has much weaker bacteria-binding activity. There is barely more bacterial binding to a surface coated with this protein than to a surface coated with rNephrin or left uncoated (coated with PBS). rNephrin is a recombinant protein containing the first two Ig domains of nephrin. It was produced using the same expression system as sMARCO and recV. Similarly to sMARCO, rNephrin contains a polyhistidine tail. Panels E and F show that bacterial binding to sMARCO is inhibited by poly(I), but not by heparin. Of these polyanions, only poly(I) is a ligand of scavenger receptors.

Next, we wanted to investigate whether MARCO binds not only heat-killed bacteria, but also living bacteria. A, Chinese hamster ovary transfectants were tested for their capability to bind FITC-labeled living *E. coli*. In this figure, the results for *E. coli* strain HB101 are shown. Cells expressing the full-length MARCO bind bacteria avidly. The binding of FITC-labeled bacteria is abolished when incubated in the presence of excess unlabeled living bacteria (not shown). MARCO-positive cells were identified as described under “Experimental Procedures.” There is no binding to control transfectants (not shown). Panels B and C show that there is avid bacterial binding to a surface coated with sMARCO, but not to a surface coated with rNephrin.
ria with high affinity. There is no significant bacterial binding to the controls, i.e. to untransfected cells (not shown) or coverslips coated with rNephrin (Fig. 7C). The binding of fluorescently labeled living bacteria is completely inhibited in the presence of a 10-fold amount of unlabeled living bacteria, indicating that binding is not a result of structural changes caused by the labeling (not shown). The binding of three different bacterial strains, wild-type E. coli K12, and E. coli strain HB101, as well as S. typhimurium LB5010, was tested. Wild-type E. coli K12 expresses the smooth form of LPS, whereas strains E. coli HB101 and S. typhimurium LB5010 express the rough form (29, 30). All these strains were recognized by MARCO (not shown), indicating that the O-side chains of LPS were not needed for bacterial recognition. It was, in fact, the case that the two mutant strains expressing the rough form of LPS were recognized by MARCO clearly better than wild-type E. coli K12, suggesting that the long O-side chains hinder MARCO-bacterial interactions.

In the last set of experiments, we used both MARCO-expressing L929 cells and sMARCO to examine whether LPS is, in fact, a ligand of MARCO. When compared with parental L929 cells (Fig. 8A), MARCO-expressing L929 cells exhibited significant binding of FITC-labeled LPS (Fig. 8B). To confirm that this binding is mediated by MARCO, an in vitro binding assay was carried out as described under “Experimental Procedures.” As shown in Fig. 8C, significant amounts of LPS precipitated with Ni-NTA beads only when sMARCO was present in the incubation solution. Thus, it was demonstrated here for the first time that LPS is a ligand of MARCO.

DISCUSSION

In this work, we have produced a recombinant, soluble form of the macrophage-specific scavenger receptor MARCO using the mammalian 293/EBNA cell expression system. MARCO is a type II transmembrane protein with N-terminal cytoplasmic and transmembrane domains. A soluble form of the protein was generated by replacing these two domains with the signal sequence of the mouse Igk chain. This modification allowed secretion of the recombinant MARCO into the culture medium, from which the protein could be purified in amounts sufficient for structural and functional analyses. This protein has made it possible for the first time to perform ligand-binding studies of MARCO in a cell-free system.

To facilitate the purification of sMARCO, we tagged the protein with a polyhistidine (His$_6$) tag at its N terminus. This modification enabled us to obtain highly purified protein with a one-step purification protocol. In our previous attempts, we tried to produce and purify an untagged form of sMARCO, but the protein tended to disappear when passed over different chromatographic matrices. We suspect that this is because of the intrinsic property of MARCO; functioning as a “molecular flypaper,” it, in fact, binds these matrices. This speculation is supported by the recent finding that MARCO is the major receptor on alveolar macrophages for the binding of unopsonized environmental particles (31). Furthermore, we have observed that if stored at +4 °C, considerable amounts of the protein are adsorbed to the walls of the storage vessel. Thus, to overcome the “stickiness” problems, one needed to develop a simple purification protocol based on affinity chromatography, and use as little affinity resins as possible.

The purified recombinant sMARCO was structurally characterized by circular dichroism analysis, thermal melting, a protease sensitivity assay, and rotary shadowing electron microscopy. All these analyses indicated that the sMARCO molecules had assembled into a collagenous triple helix. Some other recombinant collagens or their fragments have also been successfully produced using the same expression system, even though the proteins were often found to be somewhat underhydroxylated (25, 32–34).

The three chains of the sMARCO trimer were covalently associated only in a portion of the molecules, indicating that the formation of the interchain disulfide bonds is not needed for the assembly of the triple helix. Measurement of the molecular ellipticity over a range of wavelengths gave a spectrum typical for a triple-helical collagen. Analysis of the thermal stability by measuring the ellipticity at a fixed wavelength indicated a melting temperature of 44 °C. Similar, reasonably high melting temperatures have been measured for recombinant type X collagen and type X/II chimeric collagens produced in 293 cells (35, 36). MARCO contains a long continuous collagenous segment, which might be further stabilized by interactions between domains V.

The results of the trypsin-digestion experiment indicated that a major part of purified sMARCO was resistant to the enzyme digestion. The triple-helical structure of sMARCO was confirmed by imaging the purified protein electron microscopically after rotary shadowing. sMARCO appeared in these pictures as 82.7-nm long rods with globes of equal size at both ends. The length agrees well with the calculated length of 89 Gly-X-Y repeats containing the triple helix. There is one interruption within the collagenous domain of MARCO, but no bend is seen in the electron microscopic images. However, this interruption is very close to the N terminus of the collagenous domain, and may therefore not be detectable. About 50% of the triple-helical molecules were found to associate with each other via the globes, forming linear dimers as well as some linear trimers, and often also star-like molecules. An observation supporting the notion that the formation of these complexes reflects a real property of MARCO is that the stably transfected L-cells expressing the full-length MARCO have, in contrast to the parental cells, a tendency to aggregate if kept in suspension. One can speculate that if MARCO indeed associates with itself, e.g. head-to-head via domains V, this could be of signif-
iancne for the formation of a tight network of MARCO-positive cells in organs such as the spleen, where the marginal zone macrophages are filtering the passing bloodstream.

In this work, we demonstrated that MARCO not only binds heat-killed bacteria, but also living bacteria. Furthermore, it appeared that there was more avid binding to strains that express the rough form of LPS than to a strain expressing the smooth form. This finding is well in line with the knowledge that pathogenic bacteria, which express smooth LPS, are more resistant to the clearance by phagocytes compared with rough strains lacking the O-side chains (5). We also demonstrated that LPS itself is recognized by MARCO. There is at least another bacterial ligand for MARCO, because MARCO binds both Gram-negative and Gram-positive bacteria (12, 13). It is fully possible that MARCO has more than two bacterial ligands. There is, some evidence that this is the case for SR-A (37). sMARCO produced in this study will be a very useful tool in the search of ligands of MARCO.

We also produced domain V as a recombinant protein in this study, and tested its bacteria-binding activity. Previous transfection studies with transmembrane MARCO variants have indicated a crucial role for this domain in the bacteria-binding function of MARCO (13, 18). In those studies, we tested different MARCO variants lacking various regions of its extracellular domain. The extracellular part of the shortest tested MARCO variant exhibiting significant bacteria-binding activity was composed of the spacer domain, the first 8 Gly-Y repeats, and an intact domain V. The fact that this variant showed significant bacteria-binding activity was one strong support for the conclusion that domain V is of major importance for the bacteria-binding function of MARCO. This study indicated that a monomeric domain V does not have significant bacteria-binding activity. Thus, it seems that domain V has to be in a trimeric form to effectively bind to bacteria. It is also possible that the collagenuous domain does not function only as an assembly domain of MARCO, but that it directly contributes to the high-affinity bacterial binding. This notion is supported by the observation that cells expressing a MARCO variant lacking domain V but containing all other segments of the protein, have weak, but detectable bacteria-binding activity (18). If one could produce a truncated form of sMARCO containing only the collagenuous domain, it would also be of interest to compare the bacteria-binding activities of this protein and sMARCO.

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