Role of Macrophage Receptor MARCO in Host Defense

Marko Sankala

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Front cover: Rotary shadowing electron microscopy of recombinant soluble MARCO.

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You take the blue pill, the story ends...you wake up in your bed and believe whatever you want to believe.
You take the red pill, you stay in wonderland and I'll show you how deep the rabbit hole goes.

Morpheus
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. Macrophages are monocyte-derived cells which express several host-defense receptors which can be divided into two classes; those dependent on opsonizing components for recognizing pathogens, and those that can recognize pathogens directly. This latter class of receptors, pattern-recognition receptors, bind a variety of ligands including mannans and zymosan in the yeast cell wall, and various bacterial cell-wall components, such as lipopolysaccharide (LPS), lipopeptides, lipoteichoic acid (LTA) and peptidoglycans. Class A scavenger receptors are a family of pattern-recognition receptors composed of three members: Scavenger Receptor A (SR-A), Macrophage Receptor with a Collagenous structure (MARCO), and a recently identified protein Scavenger Receptor with C-type Lectin (SRCL). MARCO is a novel member of the class A scavenger receptors. It has a small intracellular N-terminal domain (I), a transmembrane domain (II), an extracellular region with a short spacer (III), a collagenous domain (IV), and a C-terminal cysteine-rich domain (V). In normal mice, this receptor is expressed only by a subset of macrophages in the marginal zone of the spleen and the medullary cord of lymph nodes, as well as in the macrophages of the peritoneal cavity. Cells transfected with MARCO cDNA avidly bind both Gram-negative and Gram-positive bacteria, but not yeast. MARCO has also been found to be a major receptor on alveolar macrophages for binding of unopsonized environmental particles, such as TiO$_2$ and Fe$_3$O$_3$. These findings strongly suggest a role for MARCO in host-defense mechanisms.

As a first part of this work, we have determined the primary structure of human MARCO and studied its spatial expression using in situ hybridization. Also, the disulfide bridge pattern of the COOH-terminal cysteine-rich domain V was determined. The work in this, and the second part of the study convincingly demonstrated that the
cysteine-rich domain V is the predominant bacteria-binding domain in MARCO. Interestingly, the corresponding domain of SR-A is still without a known function. Studies with a series of MARCO truncations containing only short segments of domain V provided information about the requirements for high-affinity bacterial binding. These studies showed that the motif RXR was identified as an essential element for high-affinity bacterial binding in these forms of MARCO.

As an important part of this study, we established the production and purification system for recombinant soluble MARCO. Analysis of the structure of the purified protein indicated that soluble MARCO forms stable elongated triple-helical molecules. We showed in functional studies that MARCO not only binds heat-killed bacteria, but also living bacteria. We also demonstrated that LPS itself is recognized by MARCO. Moreover, we have produced and purified recombinant domain V, and studied whether a surface coated with this protein supports binding of bacteria. This study indicated that compared to soluble MARCO, the monomeric domain V has low, barely detectable bacteria-binding activity. This finding highlights the importance of trimerization for the bacteria-binding activity of MARCO.

In the fourth part of this work, we show a unique role for the marginal zone macrophages in the controlling of the retention and trafficking of the marginal zone B cells. This work originated from the observation that in mice deficient in the inhibitory signaling molecule SH2-containing inositol-5-phosphatase 1, the marginal zone macrophages had migrated to the red pulp and the marginal zone B cells had disappeared. Experiments utilizing soluble MARCO demonstrated that the interaction between the marginal zone macrophages and the marginal zone B cells is mediated by MARCO on the macrophages and its unknown ligand on the B cells.

In the last part of this study, we first show using plasmon surface resonance technique that soluble MARCO binds LPS, LTA and poly(I). We detected no binding to heparin which is in line with the fact that heparin is not a ligand of scavenger receptors although it is a negatively charged macromolecule. Attempting to find novel ligands of MARCO, we then utilized the phage display technology, and searched for MARCO-binding phages from a random, complex decapptide phage library. The screening resulted in selection of novel MARCO-binding peptides. Interestingly, the peptides were found to bind to the SRCR domain of MARCO. This finding strengthens the notion that the SRCR domains constitute the biologically active part in MARCO. Current studies are directed towards finding the protein(s) that the selected peptides represent.
List of publications and manuscripts


* These authors have made equal contribution
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<th>Description</th>
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<tbody>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>AM</td>
<td>Alveolar macrophage</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guérin</td>
</tr>
<tr>
<td>CRD</td>
<td>Carbohydrate recognition domain</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CSF</td>
<td>Colony–stimulating factor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Gal</td>
<td>Galactose</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte–macrophage colony–stimulating factor</td>
</tr>
<tr>
<td>HIN</td>
<td>High in normal</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>INF</td>
<td>Interferon</td>
</tr>
<tr>
<td>LBP</td>
<td>Lipopolysaccharide binding protein</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LOX-1</td>
<td>Lectin like oxidized LDL-receptor 1</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine-rich region</td>
</tr>
<tr>
<td>LTA</td>
<td>Lipoteichoic acid</td>
</tr>
<tr>
<td>MARCO</td>
<td>Macrophage receptor with a collagenous structure</td>
</tr>
<tr>
<td>MBP</td>
<td>Mannose-binding protein</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MMR</td>
<td>Macrophage mannose receptor</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PALS</td>
<td>Periarteriolar lymphoid sheaths</td>
</tr>
<tr>
<td>PAMS</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear leukocytes</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>SAP</td>
<td>Serum amyloid protein</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecylsulfate</td>
</tr>
<tr>
<td>SHIP</td>
<td>SH2-containing inositol-5-phosphatase 1</td>
</tr>
<tr>
<td>SP</td>
<td>Surfactant protein</td>
</tr>
<tr>
<td>SR</td>
<td>Scavenger receptor</td>
</tr>
<tr>
<td>SR-CL</td>
<td>Scavenger receptor with C-type Lectin</td>
</tr>
<tr>
<td>SRCR</td>
<td>Scavenger receptor cysteine-rich domain</td>
</tr>
<tr>
<td>SREC</td>
<td>Scavenger receptor expressed by endothelial cells</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/IL-1R</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>UGRP</td>
<td>Uteroglobin-related protein</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
</tbody>
</table>
Our bodies are constantly exposed to microorganisms present in the environment. These microorganisms can use many external or internal epithelial surfaces to infect cells. As a result, all multicellular organisms have developed the ability to recognize invading microbes and to eliminate them efficiently without causing damage to self. The function of the immune system is based on two distinct recognition systems: innate and adaptive. The induction of an appropriate effector function in clonal cells is mediated by the innate immune system, while both the adaptive and innate immune responses control self/non-self discrimination. Innate immunity functions as a first line of defense for multicellular organisms. It depends upon germline-encoded receptors to recognize features that are common to many pathogens. These pattern recognition receptors (PRRs) are strategically expressed on cells that are the first to encounter pathogens during infection, such as surface epithelia, and also on all types of effector cells of the innate immune system, including antigen-presenting cells (APCs).

Class A scavenger receptors are a family of pattern-recognition receptors composed of three members: Scavenger Receptor A (SR-A), Macrophage Receptor with a Collagenous structure (MARCO), and a recently identified protein Scavenger Receptor with C-type Lectin (SRCL). SR-A 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 (SRCR). SR-A has been shown to bind a large number of polyanionic molecules, including the prototypic SR ligand, modified LDL, as well as lipopolysaccharide (LPS) and lipoteichoic acid (LTA). This 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 and instead has a long collagenous domain. In mice, MARCO expression is restricted to marginal zone macrophages in spleen, which are located between the red
pulp and the white pulp, and corresponding macrophages in lymph nodes. Furthermore, it is expressed by the macrophages of the peritoneal cavity. This expression pattern strongly suggests a role for MARCO in host-defense mechanisms.

In this thesis work, we have studied the structure and function of MARCO. In the review of literature, a brief overview of the innate immunity and antigen presenting cells is first provided. We then discuss body’s responses to LPS, a very potent inducer of innate immunity. This is followed by an overview of the Toll-like receptors. Finally, the literature concerning scavenger receptors, particularly the class A family, is reviewed.
Review of literature

**Innate immunity**

Infectious diseases are caused by microorganisms. At the moment we can recognize four broad categories of disease-causing microorganisms or pathogens: viruses, bacteria, pathogenic fungi and parasites. Innate immunity functions as a first line of defense against these pathogens. It appeared early in evolution and is believed to have predated the adaptive immune response on several grounds. First, the innate host defenses are found in all multicellular organisms, whereas the adaptive immunity is found only in vertebrates. Secondly, the innate immune recognition distinguishes self from non-self perfectly. Third, the innate immune system uses receptors that are ancient in their lineage, whereas the adaptive immunity uses specific antibodies and T cell receptors. In order to function efficiently, immune system must be able to recognize and destroy a wide variety of pathogens without causing damage to self. The problem is the enormous heterogeneity of pathogens and limited number of different recognition molecules in the genome. That is why these few molecules have to be able to recognize a vast variety of molecular structures associated with pathogens. These structures must be shared by large groups of pathogens, and thus have to be specific molecular patterns rather than particular structures. This implies that these molecular patterns must be conserved products of microbial metabolism (for example, the molecular pattern of lipopolysaccharide [LPS] is common to all Gram-negative bacteria) and they are essential for microbial survival. Mutations or loss of these patterns are either lethal for that class of microorganisms, or they greatly reduce their adaptive fitness (Medzhitov & Janeway 1997; Janeway et al. 2001; Janeway & Medzhitov 2002).

Conserved pathogen-associated molecular patterns (PAMS) are targets for many recognition molecules in innate immunity. Receptors of the innate immune system that recognize PAMPs are called pattern recognition receptors (PRRs). PRRs are
strategically expressed on cells that function as a first line of defense, such as surface epithelia and antigen-presenting cells such as macrophages and dendritic cells. Currently we can distinguish three functional classes of receptors (Table I): 1) endocytic receptors, such as cellular C-type lectins, scavenger receptors (SRs) and integrin CD11b,c:CD18 may facilitate opsonization and phagocytosis. 2) Secreted proteins, which include mannose-binding protein (MBP), C1q, pulmonary surfactant proteins A and D (SP-A, SP-D), and C-reactive protein (CRP). They promote opsonization for phagocytosis and the complement system to destroy secreted protein-bound pathogens. 3) Signaling receptors, such as the Toll-like receptors (TLR), which can activate intracellular signals that induce many immune response genes. PAMP recognition can directly activate effector mechanisms of innate immunity, such as phagocytosis, induction of the synthesis of antimicrobial peptides, and induction of nitric oxide synthase in macrophages. Additionally, PAMPs trigger many endogenous signals leading to secretion of inflammatory cytokines, such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, type I interferon (IFN), major effector cytokines and many chemokines. Most importantly, recognition of PAMPs by PRRs induces the expression of co-stimulatory molecules on APCs (Medzhitov & Janeway 1997; Janeway et al. 2001; Janeway & Medzhitov 2002).

Table I. Some PRRs and their ligands.

<table>
<thead>
<tr>
<th>PRR</th>
<th>Protein/domain family</th>
<th>Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Secreted PRRs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBP, C1q, SP-A, SP-D</td>
<td>C-type lectin</td>
<td>Bacterial and viral carbohydrates</td>
</tr>
<tr>
<td>CRP, SAP</td>
<td>Pentraxins</td>
<td>Phosphorylcholine on microbial membranes</td>
</tr>
<tr>
<td>LBP</td>
<td>Lipid-transfer protein family</td>
<td>LPS</td>
</tr>
<tr>
<td><strong>Cell-surface PRRs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD14</td>
<td>Leucine-rich repeats</td>
<td>LPS, peptidoglycan</td>
</tr>
<tr>
<td>MMR</td>
<td>C-type lectin</td>
<td>Terminal mannose residues</td>
</tr>
<tr>
<td>SRs</td>
<td>Scavenger receptor cysteine-rich domain</td>
<td>LPS, dsRNA, oxidized LDL, anionic polymers</td>
</tr>
<tr>
<td>MARCO</td>
<td>Scavenger receptor cysteine-rich domain</td>
<td>LPS, bacterial cell walls</td>
</tr>
<tr>
<td><strong>Signaling PRRs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLRs</td>
<td>Leucine-rich repeats</td>
<td>LPS, lipoteichoic acids, peptidoglycan, lipoarabinomannan</td>
</tr>
</tbody>
</table>

SAP, serum amyloid protein, LBP, lipopolysaccharide binding protein, MMR, macrophage mannose receptor. Adapted from (Medzhitov 2001).


**Activation of adaptive immune response**

Antigen processing cells, which have recognized pathogens, process the same pathogen into specific pathogen-derived antigens and present them with major histocompatibility complex (MHC) II to CD4+ T cells (Figure 1). T cells will be activated only when they recognize signals both from peptide antigens and the expression of pathogen-induced co-stimulatory molecules (B7) on the surface of APCs. Effector cytokines induced by the pathogen instruct the activated T cells to differentiate into a particular effector cell type called helper T (T\(_{H1}\) or T\(_{H2}\)). Activated T cells then deliver an inducible signal (such as CD40 ligand) to the target cell in an antigen-specific manner: the target cells of T\(_{H1}\) and T\(_{H2}\) being macrophages and B cells, respectively. These activated B and T cells in turn cooperate with activated macrophages to enhance destruction of intra- and extracellular pathogens (Medzhitov & Janeway 1998; Janeway & Medzhitov 2002).

![Figure 1. Recognition of pathogen by PRRs activates adaptive immunity. Adapted from (Medzhitov & Janeway 1997).](image-url)
Macrophages

All the cellular elements of blood derive ultimately from the same progenitor or precursor cells - the hematopoietic stem cells in the bone marrow. The myeloid progenitor is the precursor of the granulocytes, macrophages, dendritic cells, and mast cells of the immune system. Macrophages are one of the three types of phagocyte in the immune system and are distributed widely in the body tissues, where they play a critical part in innate immunity. They are the mature form of monocytes, which circulate in the blood and differentiate continuously into macrophages upon migration into the tissues. Differentiation of a monocyte into a tissue macrophage involves a number of changes: the cell enlarges five- to tenfold, its intracellular organelles increase in both number and complexity, it acquires increased phagocytic ability, produces higher levels of lytic enzymes, and begins to secrete a variety of soluble factors. There are lots of growth and differentiation factors involved in maturation of monocytes to macrophages, e.g. colony–stimulating factor 1 (CSF–1), granulocyte–macrophage CSF (GM–CSF) and interleukin 3 (IL–3), as well as inhibitors such as macrophage inflammatory protein 1α (MIP1α) (Gordon et al. 1995; Janeway et al. 2001).

Macrophages are dispersed throughout the body. Some take up residence in particular tissues becoming fixed macrophages, whereas others remain motile and are called free, or wandering, macrophages. Free macrophages move by amoeboid movement throughout the tissues. Fixed macrophages serve different functions in different tissues and are named to reflect their tissue location: Alveolar macrophages in the lung; Histiocytes in connective tissue; Kupffer cells in the liver, Mesangial cells in the kidney, and Microglial cells in the brain. Macrophages are involved in many immune and nonimmune activities. These different activities include tissue maintenance, immune regulation and pathogen control. Tissue maintenance is composed of both tissue repair and modeling activities. In bones, osteoclasts are responsible for remodeling. Other examples of tissue maintenance are splenic macrophages, which mediate erythroleukophagocytosis, and Kupffer cells that manage gut-absorbed endotoxin and erythrophagocytosis in the neonatal liver. Macrophages play a central role in immune-regulation acting as an accessory cell coordinating the interaction of T and B cells during antigen presentation. Finally, the antipathogenic properties of macrophages are illustrated by their antibacterial, -parasitic, -fungal, and – viricidal properties. Macrophages may also have a role in other functions, such as wound repair (Turpin & Lopez-Berestein 1993).
Marginal zone macrophages

The spleen is a discriminatory filter which removes aged erythrocytes and platelets from the circulation through phagocytic cells (macrophages) in a specialized section of the splenic venous compartment, the venous sinuses of the red pulp. Another major component of the splenic parenchyma is the white pulp, which consists of lymphoid sheaths organized around branches of the splenic artery (periarteriolar lymphoid sheaths, PALS). It is in the red pulp that blood filtration takes place, whereas the white pulp is involved in the specific immunological defense against bloodborne antigens. The periphery of the PALS, at the boundaries between the white and the red pulp, is termed the marginal zone. In this marginal zone part of the arterial blood stream opens into sinuses, and it is here that antigens come into contact with a set of conspicuous cell types with peculiar features. We can distinguish several types of macrophages in the marginal zone, such as marginal zone macrophages and marginal metallophilic macrophages (Kraal 1992; Janeway et al. 2001).

Marginal metallophilic macrophages are strategically positioned along the borders of the marginal sinus. They can be identified with the monoclonal antibody MOMA-1 (Kraal & Janse 1986), which recognizes the sialoadhesin receptor. They have high activity for nonspecific esterase and low phagocytic activity, and they express receptors for sialic acid-containing glycoconjugates. The first characteristic indicates a role in the degradation of incoming material (e.g., endotoxin); the latter suggests a role in the interaction with lymphocytes (Kraal 1992).

Marginal zone macrophages form a ring of two or three layers dispersed throughout the marginal zone. They are big cells with long cell processes and appear to have close contact with the surrounding marginal zone B cells. They are highly phagocytic cells and bind neutral polysaccharides (Humphrey & Grennan 1981). Marginal zone macrophages can be identified in mouse with the antibody ER-TR9 (Dijkstra et al. 1985). Recently the antigen for ER-TR9 has been identified as a murine homolog of human dendritic cell specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) (Geijtenbeek et al. 2002; Kang et al. 2003). This mSIGNR1 belongs to C-type lectins which are important pathogen recognition receptors. All ER-TR9-positive marginal zone macrophages also express MARCO, but there are small numbers of MARCO-positive macrophages which do not express SIGNR1 (Elomaa et al. 1995).
Dendritic cells

The dendritic cell acquired its name because it is covered with a maze of long membrane processes resembling dendrites of nerve cells. They are bone marrow-derived cells of both lymphoid and myeloid stem cell origin that populate all lymphoid organs including the thymus, spleen and lymph nodes, as well as nearly all nonlymphoid tissues and organs (Vandenabeele & Wu 1999). Most dendritic cells process and present antigen to T\(_H\) cells. These cells can be classified based on their location: Langerhans cells found in the epidermis and mucous membranes; Interstitial dendritic cells which populate most organs; Interdigitating dendritic cells present in T-cell areas of secondary lymphoid tissue and the thymic medulla; Circulating dendritic cells including those in the blood. The dendritic cells in each of these locations have morphologic and functional differences. They express high levels of both class II MHC and co-stimulatory molecule B7. For this reason, they are more potent antigen-presenting cells than macrophages and B cells. After capturing antigen in the tissues by phagocytosis or endocytosis, dendritic cells migrate into the blood or lymph and circulate to various lymphoid organs where they present the antigen to T lymphocytes (Lipscomb & Masten 2002). Following the encounter of antigen/pathogen they undergo phenotypic and functional changes that transform them from antigen-capturing cell to antigen presenting cells. During this process, dendritic cells also undergo profound cytoskeleton rearrangements. Immature dendritic cells are slightly adherent low motile cells with organized actin-based cytoskeleton, while matured dendritic cells are non-adherent cells with disassembled cytoskeleton (Winzler et al. 1997).

Induction of innate immunity

Many components of microorganisms induce innate immune responses. However, we will below concentrate on the effects of bacterial lipopolysaccharide (LPS) since it is among the most potent inducers of these responses, and its effects are most extensively studied. These studies have in recent years also revealed the importance of the Toll-like receptors for the induction of innate immunity. The literature concerning the Toll-like receptors will be reviewed. However, one should keep in mind that an organism is exposed during infection to multiple inducers of the innate immunity, and that the outcome of these responses depends on the interplay between different innate immunity receptors. Currently very little is known about the interplay between these systems.
Bacterial lipopolysaccharides

Bacterial lipopolysaccharides are the major outer surface membrane components present in all Gram-negative bacteria (for example *Escherichia coli* and *Neisseria meningitidis*) and act as extremely strong stimulators of innate or natural immunity. They are extremely heat-stable amphiphilic molecules composed of a predominantly lipophilic region, lipid A and covalently linked hydrophilic poly- or oligosaccharide portion. The polysaccharide region is commonly subdivided into the terminal O-specific chain and the core region most proximal to lipid A (Figure 2). According to preferential carbohydrate compositions in the core structure, an inner and an outer core region are commonly distinguished. The O-specific chain is characterized by extremely high structure variability even within a given bacterial species, which constitutes the chemical basis for the serological classification of individual wild-type bacterial strains according to the O-antigenic determinants. Based on the characteristic colony morphology, bacteria synthesizing O-specific chains are called the smooth (S)-form, whereas bacteria synthesizing LPS without O-specific chains are termed as rough (R)-mutants. Rough mutants are able to grow and multiply *in vitro*, showing that the O-chain is not necessary for viability. However, in tissues or body fluids, pathogenic enterobacteria can only persist and survive if they express an O-specific chain, which in this case protects the bacteria from uptake by phagocytes and the attack of serum complement. Yet, it has also been found that there are many wild-type species of pathogenic Gram-negative bacteria lacking the O-specific chain in the LPS structure. In general the combined lipid A and inner core substructures of LPS form an extremely charged region of the bacterial outer membrane, which is a target for a wide variety of cationic antibiotics, positively charged host-defense peptides and proteins (Alexander & Rietschel 2001; Van Amersfoort *et al.* 2003).

![Figure 2. General structure of LPS from Gram-negative bacteria.](image)

All forms of LPS consist of the membrane-anchoring lipid A domain and a covalently linked polysaccharide or oligosaccharide portion. Adapted from (Van Amersfoort *et al.* 2003).
Lipid A domain is the most conserved structure in LPS, and therefore it functions as a PAMP for infection by Gram-negative bacteria. Small amounts of bacteria-derived lipid A rapidly activates humoral and cellular defense. Humoral factors comprise complement, antibodies, and acute-phase proteins, whereas the cellular line of defense comprises mononuclear cells and neutrophils. In mammalian species, this early activation of innate immunity also induces the subsequent recruitment of adaptive, highly specific immune responses via the selection and clonal expansion of pathogen-specific T- and B-lymphocytes. In serum LPS binds to an acute-phase protein called lipopolysaccharide-binding protein (LBP) (Wright et al. 1989). LBP catalyses the transfer of monomerized LPS to CD14 present either in the glycosylphosphatidylinositol-anchored (GPI) form (mCD14) on the phagocyte surface or in the soluble form (sCD14) in the extracellular space (Wright et al. 1990). CD14 is not a transmembrane protein, and therefore it was speculated soon after its discovery that another receptor is needed to initiate the signaling. Indeed, TLR-4, which forms a complex with another protein called MD-2 (Shimazu et al. 1999), was identified as such a receptor. Binding of LBP-LPS-CD14 to TLR-4*MD-2 complex leads to rapid and coordinated activation of various intracellular signaling pathways including activation of the major mitogen-activated protein kinase (MAPK) cascades and translocation of transcription factor NF-κB. There are also other phagocyte surface proteins contributing to signal transduction, but their role is not yet clear (Alexander & Rietschel 2001; Van Amersfoort et al. 2003).

Sepsis is an infection in the bloodstream. Septic diseases represent the prevalent complications in intensive care units affecting more than 50% of all intensive care patients. Normally the release of TNF-α by macrophages induces local protective effects, but can have damaging effects when released systemically. In local release TNF-α acts on blood vessels, especially venules, to increase blood flow, to increase vascular permeability to fluid, proteins and cells, and to increase endothelial adhesiveness for leukocytes and platelets. Later, blood clots form in the small vessels, preventing spread of the infection via the blood, and the accumulated fluid and cells drain to regional lymph nodes where the adaptive immune response is initiated. If the infection spreads to the bloodstream, the systemic release of TNF-α into the blood by macrophages in the liver and spleen causes similar effects on all small blood vessels. The result is shock, disseminated intravascular coagulation with depletion of clotting factors and consequent bleeding, multiple organ failure, and frequently death. These
effects require the presence of TLR-4 on macrophages (Alexander & Rietschel 2001; Van Amersfoort et al. 2003).

**Toll-like receptors**

The first member of this still growing family of innate immune receptors was originally described in *Drosophila* as a type I transmembrane protein crucial for dorsal-ventral patterning of fly embryos. It was later also found to play a key role in the antifungal immunity of the imago (Hashimoto et al. 1988; Lemaitre et al. 1996). The Toll signaling pathway in adult flies induces the production of several antimicrobial peptides that contribute to the fly’s defense against infection. Toll proteins have three domains: the leucine-rich region (LRR) and the cysteine-rich domain in the extracellular part and a Toll/IL-1R (TIR) homology domain in the intracellular part of the receptor (Gay & Keith 1991). A recent study indicates that the Toll pathway is required for resistance to Gram-positive bacterial infection in addition to fungal infections (Rutschmann et al. 2002). The *Drosophila* response to Gram-negative bacterial infection is controlled by a distinct pathway, which was defined by the mutation in the *imd* (immune deficient) gene. *Imd* mutants have a profound defect in resistance to Gram-negative bacterial pathogens, while remaining essentially normal with regard to fungal and Gram-positive infection (Lemaitre et al. 1995). After identification of *Drosophila* Toll, a family of proteins structurally related to this protein was identified, collectively referred to as the Toll-like receptors. To date, the TLR family is known to consist of 10 members (TLR1-10). TLR ligands are quite diverse in structure and origin. Most TLR ligands are PAMPs that signal the presence of infection. Many, and perhaps all, individual TLRs can recognize several structurally unrelated ligands, but some TLRs require accessory proteins to recognize their ligands (Figure 3). Although the actual mechanism of ligand recognition is still not known, available evidence indicates that mammalian TLRs recognize their ligands by direct binding and therefore function as PRRs (Takeda et al. 2003).
Figure 3. Different TLRs and ligands they recognize. It is currently thought that TLR-3, -4, -5, -7 and –9 transport their signals by forming homodimers after interacting with their ligands. TLR-2 is forming a heterodimer with another TLR (TLR-1, -6 and most likely TLR-10. For TLR-4, MD-2 is also required for ligand recognition. Adapted from (Akira 2003).

The expression pattern of the TLRs varies extensively. Monocytes and macrophages express mRNA for most TLRs except TLR3, which is expressed only in NK cells (Hornung et al. 2002). TLR2 and TLR4 have similar expression pattern and are normally found in PMN, monocytes, macrophages and dendritic cells (Muzio et al. 2000a; Muzio et al. 2000b). Both receptors are also present on various other cell types including epithelial and endothelial cells (Zhang et al. 1999; Cario et al. 2000; Faure et al. 2000). Ectopic overexpression of TLR4, the first mammalian TLR identified, was shown to cause induction of the genes for several inflammatory cytokines and costimulatory molecules (Medzhitov et al. 1997). Later TLR4 was shown to be involved in the recognition of LPS, where it functions as the signal-transducing receptor for LPS (Poltorak et al. 1998; Hoshino et al. 1999; Qureshi et al. 1999). This discovery was made by positional cloning of the lps gene in the LPS-non-responsive C3H/He mouse strain (Poltorak et al. 1998; Qureshi et al. 1999), and was confirmed in tlr4 knockout mice (Hoshino et al. 1999). Recognition of LPS requires several accessory molecules, such as LBP, CD14 and MD-2 as discussed in chapter Bacterial lipopolysaccharides. B cells express on their surface a receptor called RP105 that is also involved in LPS recognition (Miyake et al. 1995). RP105 is a LRR-containing protein that has similar kind of ectodomain as TLR4. However, RP105 lacks the TIR domain and instead has a short cytoplasmic region with a tyrosine-phosphorylation motif. Ligation of RP105 leads to activation of SRC-family tyrosine kinases. RP105 is associated via its ectodomain with MD-1, a protein related to MD-2 that is required for RP105 function (Miura et al. 1998; Miyake et al. 1998). Of the mammalian TLRs, TLR2 recognizes the largest group of ligands, such as peptidoglycan (Schwandner et al. 1999; Takeuchi et al. 1999) and bacterial lipoproteins (Aliprantis et al. 1999; Brightbill
et al. 1999; Takeuchi et al. 2000). This unusually broad range of ligands recognized by TLR2 is explained, in part, by cooperation between TLR2 and at least two other TLRs: TLR1 and TLR6 (Ozinsky et al. 2000; Takeuchi et al. 2001).

Activation of signal transduction pathways by TLRs leads to the induction of various genes whose products function in host defense, including inflammatory cytokines, chemokines, MHC and costimulatory molecules. Mammalian TLRs also induce multiple effector molecules, such as inducible nitric oxide synthase and antimicrobial peptides, which can directly destroy microbial pathogens (Thoma-Uszynski et al. 2001). TLRs can activate adaptive immunity by inducing maturation of dendritic cells. Mature dendritic cells express high levels of MHC and co-stimulatory molecules (CD80 and CD86) and migrate to draining lymph nodes, where they present pathogen-derived antigens to naïve T cells (Banchereau & Steinman 1998). TLRs also induce expression by dendritic cells of various cytokines, including IL-12, which directs T_{H1} cell differentiation into T_{H1} effector cells (Akira et al. 2001).

**Scavenger receptors**

Scavenger receptors are a family of cell surface glycoproteins able to bind modified lipoproteins, such as oxidized and acetylated LDLs (OxLDL and AcLDL) (Peiser et al. 2002a). They were first discovered in 1979, during attempts to learn how cholesterol from low density lipoproteins (LDL) accumulates in macrophages of the atherosclerotic plaques (Goldstein et al. 1979). Originally known as acetylated LDL receptors, they are now termed scavenger receptors. It was quickly noted that these receptors have broad ligand binding ability leading to their description as ‘molecular flypaper’. Almost all the characterized ligands are polyanionic molecules, although many polyanions fail to bind (Krieger & Herz 1994). Table II lists some of the known ligands for the various SRs. All SRs are related by biological property rather than by sequence homology, and are categorized in six different classes. Class A SR was the first SR to be cloned, and it has six family members. SR-AI (Kodama et al. 1990) and II (Rohrer et al. 1990) are two naturally occurring isoforms. SR-AIII (Gough et al. 1998) is an alternative splice variant. MARCO is classified as a scavenger receptor because of the structural similarities (Elomaa et al. 1995). This class includes also novel collagenous receptors SR-CLI and II (Nakamura et al. 2001b; Nakamura et al. 2001a). An independent group cloned the same molecule and termed it CL-P1 (Ohtani et al. 2001). The class B SRs consist of CD36 (Endemann et al. 1993), SR-BI (Acton et al. 1994) and II (Webb et al. 1997), CLA-1 (Cao et al. 1997; Murao et al. 1997), the human homologue of SR-BI,
and *drosophila* receptor *Croquemort* (Franc *et al*. 1996). The *drosophila* SR-CI (Pearson *et al*. 1995) belongs to class C, and CD68/macrosialin (Holness *et al*. 1993) forms class D. LOX-1 (lectin like oxidized LDL-receptor 1) (Sawamura *et al*. 1997) belongs to class E, and scavenger receptor expressed by endothelial cells I (SREC-I) (Adachi *et al*. 1997) and II (Ishii *et al*. 2002) belong to class F. There are also some new receptors, such as the scavenger receptor for phosphatidylserine and oxidized lipoprotein (SR-PSOX) (Shimaoka *et al*. 2000) and FEEL-1 (fasciclin, EGF-like, laminin-type EGF-like, and link domain-containing scavenger receptor-1) (Adachi & Tsujimoto 2002).

Here, we will review the structure and function of the class A SRs (Figure 4). This does not, however, implicate that the other SRs are of no significance for innate immunity.

**Table II. Ligand binding properties of the SR family of molecules.**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Class A</th>
<th>Class B</th>
<th>Class C</th>
<th>Class E</th>
<th>Class F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SR-AI, -II</td>
<td>MARCO (CL-P1)</td>
<td>SR-CLI, -II</td>
<td>SR-BI</td>
<td>CD36</td>
</tr>
<tr>
<td>oxLDL</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>+</td>
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<tr>
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<td>–</td>
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<td>ND</td>
<td>+</td>
<td>ND</td>
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<td>ND</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>malBSA</td>
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<td>ND</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>BSA</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Fucidin</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Dextran sulphate</td>
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<td>ND</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
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<tr>
<td>Chondroitin sulphate</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>PolyI/PolyG</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PolyC/PolyA</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Phosphatidylinerine</td>
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<td>ND</td>
<td>ND</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Apoptotic cells</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Gram-negative bacteria</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Gram-positive bacteria</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Asbestos</td>
<td>+</td>
<td>LPS, LTA</td>
<td>ADG modified proteins, β-aminoid</td>
<td>+</td>
<td>LPS, LTA</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Collagen, <em>Plasmodium</em> infected erythrocytes, thrombospondin</td>
</tr>
</tbody>
</table>

* The known ligand binding properties of the class A-F SRs are shown, except for CD68/macrosialin (class D) as the only known ligand is OxLDL. + denotes receptor binding of a particular ligand either by direct binding or the ability to inhibit mLDL endocytosis; – symbolizes the molecule is unable to bind a particular receptor; ND indicates no reports to date of a receptor–ligand interaction. Adapted from (Peiser & Gordon 2001).
Figure 4. Scavenger receptors implicated in microbial recognition. Adapted from (Peiser et al. 2002a).

**SR-AI and II**

**Structure and expression**

Type I and II receptors are mainly expressed in macrophages and related cells. They were first isolated from bovine lung, but have been found in mice (Ashkenas et al. 1993), humans (Matsumoto et al. 1990), and rabbits (Bickel & Freeman 1992). Type I and type II receptor proteins are two naturally occurring isoforms (Rohrer et al. 1990), which are alternative splice variants of the same gene found on chromosome 8p22 in humans (Matsumoto et al. 1990). Recently, a third alternative splice variant, type III, has been described. SR-AIII is not functional, and is trapped in the endoplasmic reticulum. It displays dominant negative properties, and thus may be one post-translational control mechanism for SR-AI and II (Gough et al. 1998). Type I, II and III receptors differ from each other with regard to their C-terminal region: SR-AI expresses a conserved 110-amino acid scavenger receptor cysteine rich domain (Resnick et al. 1996; Hohenester et al. 1999), while SR-AII and SR-AIII have a short or truncated C-terminal region, respectively. No functional difference between type I and type II has been described. However, recent data suggested, but did not confirm, differential binding...
of bacteria (Peiser et al. 2000). Type I and II receptors are constitutively present in
resident macrophages including Kupffer cells of the liver, as well as in peritoneal,
alveolar and other macrophages (Naito et al. 1991; Naito et al. 1992). Epidermal
Langerhans cells and dendritic cells express low or undetectable levels. In normal brain
type I and type II proteins are expressed in the perivascular macrophages surrounding
arterioles named MATO cells, but not in the microglial cells (Mato et al. 1996). Type I
and type II receptors are also expressed in sinusoidal endothelial cells of the liver and
the adrenal gland (Naito et al. 1991), and in high endothelial cells of postcapillary
venules in lymph nodes (Geng & Hansson 1995). Exposure of macrophages to LPS has
been shown to influence the expression of SR-A in a species-dependent manner. In
human monocyte-derived macrophages, LPS exposure decreased SR activity and
downregulated SR-A message levels (Van Lenten et al. 1985; van Lenten & Fogelman
1992). Paradoxically, in the murine system, the opposite appears to occur (Fitzgerald et
al. 2000): endotoxin induced SR-A mRNA through a posttranscriptional mechanism
that promoted either message maturation or export. Interestingly, TNF-α treatment of
mouse cells could not mimic the effects of LPS, indicating that the increase was not the
result of autocrine/paracrine production of this cytokine. In humans downregulation of
SR-A is mediated via TNF-α (van Lenten & Fogelman 1992). One possible explanation
is that the regulatory regions of the human and mouse genes may differ in the presence
of response elements for endotoxin-induced transcription factors.

Type I and II receptors are type II trimeric transmembrane glycoproteins, which
can be divided into six distinct domains (see figure 4) (Kodama et al. 1990). The first
50 N-terminal residues of SR-IA and II comprise the cytoplasmic domain which is
followed by a 25-residue long transmembrane region, a 75-residue spacer domain,
which may be N-glycosylated, a 121-residue α-helical coiled coil domain, a 69-residue
long collagenous region, and the C-terminus called the SRCR (scavenger receptor
cysteine-rich) domain. Electron microscopy using rotary shadowing and negative
staining showed that an extremely flexible hinge joins the coiled-coil and collagenous
domains. The angle between these domains varies from 0 to 180° (Resnick et al. 1996).

Domain I compromises the SR-A cytoplasmic tail and it does not contain any of
the classical motifs that are present in other endocytic receptors. It is suggested that the
secondary structure of the tail might be important, and more specifically, a β turn has
been implicated (Fong & Le 1999). The domain does contain two potential protein
kinase C (PKC) phosphorylation sites (Ashkenas et al. 1993), and the receptor can be
coimmunoprecipitated with Lyn kinase suggesting receptor signaling (Miki et al. 1996). The tail also lacks any known conserved motifs required for phagocytosis, though the downstream events following receptor ligation by other archetypal phagocytic receptors are now just beginning to be understood (Aderem & Underhill 1999).

Domain IV forms an $\alpha$-helical coiled coil structure on the basis of 23 heptad repeats. This domain is important in receptor trimerization and dissociation from the ligand in endosomes (Acton et al. 1993; Doi et al. 1994). Circular dichroism spectroscopy analyses of recombinant mutants of human SR-A have demonstrated that the seven-residue sequence Ile$^{173}$-Ser$^{179}$ is essential for receptor oligomerization (Frank et al. 2000). There are five sites for N-linked glycosylation in this domain. A pH-dependent conformational change within this domain is believed to be responsible for intracellular dissociation of endocytosed ligands and recycling of the receptor (Doi et al. 1994).

The collagenous domain is characteristic for the class A SRs and is present in all members of the class. In case of SR-AI and II, it is made up of 23 Gly-X-Y repeats, where X and Y can be any amino acid, although lysine and proline make up over 50% of the amino acids in the Y position. The repeats are thought to be neutral or positively charged at a physiological pH. Unpaired lysine residues at the outer edge of the helix would be available for intermolecular interactions with negatively charged ligands (Acton et al. 1993). Studies with a series of truncated and point-mutant variants have revealed that the carboxyl-terminal 22 amino acids contain the site for AcLDL recognition (Doi et al. 1993). Mutation of Lys$^{337}$ of the bovine protein abolished all AcLDL binding. However, a wider structure-activity study of the collagenous domain of the rabbit receptor indicated that residues distinct from the terminal 22 amino acids also affect the binding of modified lipoprotein (Andersson & Freeman 1998). The apparent discrepancy between these data sets may be a result of the temperature at which the binding studies were performed. Ligand recognition apparently requires complex conformational interactions independent of the terminal collagenous sequences, which may explain the failure of short peptide models to mimic the properties of the entire domain (Anachi et al. 1995; Mielewczyk et al. 1996).

The SRCR domain is an ancient and highly conserved domain found in many proteins. The SRCR domain sequences can be divided into two groups, A and B, based primarily on the differences in the spacing pattern between their cysteine residues. All of the group A domains contain six cysteines, while most of those in group B have eight cysteines (Resnick et al. 1994). More than 50 SRCR superfamily proteins including
CD6 are reported. Because it has not so far been shown to influence the binding of modified lipoproteins, the presence of the SRCR-like motifs is not predictive of SR activity. The precise role of the SRCR domains in the various molecules of this group is unclear, except for the CD6 molecule. CD6, which is predominantly expressed on T cells, interacts via the SRCR domain with the adhesion molecule ALCAM (Whitney et al. 1995).

**Role in innate immunity**

The first suggestion that SR-A may play a role in innate immunity came from studies examining the binding of LPS and LTA to the receptor. It was shown that ReLPS and lipid IVₐ forms of bacterial LPS were able to compete for the uptake of the SR ligand AcLDL *in vitro*, and that SR ligands could reduce the clearance of LPS from the circulation *in vivo* (Hampton et al. 1991; Ashkenas et al. 1993). Binding studies with a secreted form of bovine SR-A showed binding with a number of Gram-positive bacteria, including *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Listeria monocytogenes* (Dunne et al. 1994). Interaction between bacteria and SR-A could be inhibited by LTA, suggesting that binding was probably through LTA (Greenberg et al. 1996). It was found that both the size and the distribution of the negative charge of the side chain phosphate groups of LTA determined the capacity and strength of binding. Similarly it was shown that binding of LPS by an undefined scavenger receptor on Kupffer cells and endothelial cells depends on anionic groups in the lipid A moiety (Shnyra & Lindberg 1995). It has been shown in other studies that SR-A can also bind other Gram-positive bacteria, such as *Enterococcus faecalis* and *Mycobacterium tuberculosis* (Greenberg et al. 1996; Zimmerli et al. 1996). All of these data suggest that SR-AI and II can function as pattern recognition molecules. The expression pattern of SR-A is also consistent with a role in host defense.

Analysis of the SR-A−/− mice in response to infection has provided the most definitive data on a role of SR-A in innate immunity as a possible pattern recognition receptor. SR-A−/− mice are more susceptible to Gram-positive bacteria, such as *Listeria monocytogenes* and *Staphylococcus aureus*, than wild-type mice (Suzuki et al. 1997; Thomas et al. 2000). Compared to controls, the SR-A-deficient animals were also more sensitive to DNA virus, herpes simplex virus type 1. Knockout animals also had increased bacterial burdens in liver and spleen (Suzuki et al. 1997). Neutrophils from the SR-A-deficient mice displayed normal killing of *S. aureus*, yet the animals died from disseminated infection, suggesting the importance of macrophages in clearance of
bacteria (Thomas et al. 2000). The involvement of SR-A in mediating the in vivo effects of LPS has also been studied by using the knockouts. It was shown that mice primed with Bacillus Calmette-Guérin (BCG) are more susceptible to LPS-induced shock, producing higher levels of the proinflammatory cytokines TNF-α and IL-6 than similarly infected wild type mice (Haworth et al. 1997). In contrast to these results, Kobayashi et al. demonstrated that SR-A⁻/⁻ mice are highly insensitive to endotoxin shock and produced only a small amount of IL-1 (Kobayashi et al. 2000). One explanation for the different response may be the used method; Haworth et al. primed the mice with viable BCG, while Kobayashi et al. injected LPS to unprimed mice.

Macrophages isolated from SR-A knockout mice showed that nonopsonic phagocytosis of Neisseria meningitidis is almost exclusively mediated via SR-A. Interestingly, it was also shown that SR-A binds a Neisseria meningitidis strain which is lacking LPS and that SR-A was not required for cytokine production (Peiser et al. 2002b). SR-A has also been shown to bind bacterial CpG DNA, but is not required for TNF-α release, unlike TLR (Zhu et al. 2001).

There is a large body of evidence suggesting a role for SRs in the downregulation of the inflammatory response. The transcription of some of these cytokines, like TNF-α, and production of nitric oxide, can be inhibited by OxLDL (Yang et al. 1996; Matsuno et al. 1997). NF-κB, Ca²⁺ signaling and PIP₂ have also been implicated in this suppression via SRs (Schackelford et al. 1995). Nitric oxide production can also be inhibited by maleylated BSA and phosphatidylserine-containing liposomes, but not by AcLDL or native LDL. The inhibition occurs probably through LPS-induced tyrosine phosphorylation of a 41-kDa protein (Matsuno et al. 1997).

**Phagocytosis**

Several approaches have been used to examine the ability of SR-AI and II to mediate phagocytosis of bacteria or apoptotic cells. SR-A mediated phagocytosis of Gram-negative and Gram-positive bacteria has been demonstrated (Peiser et al. 2000; Thomas et al. 2000). To study the role of SR-A in bacterial uptake directly, FACS-based in vitro assay with different strains of E. coli and S. aureus binding to control and SR-A⁻/⁻ macrophages was used. Results showed that phagocytosis via the receptor in vitro was dependent on a number of criteria including the source of macrophages, culture conditions, and the strain of bacteria used. Bone marrow culture-derived macrophages from SR-A⁻/⁻ mice ingested 30-60% fewer E. coli DH5α, but 70-75% fewer E. coli K1 (Peiser et al. 2000). The efficient phagocytosis of cells that die by apoptosis is an
important process. There is evidence for SR-A being a receptor for the recognition and internalization of apoptotic thymocytes. Thymic and peritoneal macrophages from SR-A<sup>−/−</sup> mice ingested approximately 50% fewer apoptotic cells than control 129/ICR macrophages (Platt et al. 1996). The extent of inhibition was comparable to that reported for other candidate phagocytic receptors (Platt et al. 1998) – it is increasingly apparent that binding and ingestion are complex interactions that require the activity of more than a single receptor. Analyses of apoptotic thymocyte clearance in vivo failed to reveal a significant phenotype consistent with impaired clearance (i.e., an accumulation of apoptotic corpses) (Platt et al. 2000). The most likely explanation is receptor redundancy. The cell biology of SR-A-mediated phagocytosis following receptor ligation is not understood and the endocytic trafficking of the ingested particles is unknown.

**Adhesion**

SR-A-mediated adhesion may have important physiological roles, particularly at sites of tissue inflammation. An adhesive capacity of SR-A was shown when a monoclonal antibody that could block cation independent adhesion of macrophages to tissue culture plastic in the presence of serum was discovered to be directed against SR-A (Fraser et al. 1993). Peritoneal macrophages from SR-A<sup>−/−</sup> mice displayed delayed adherence and spreading in vitro (Suzuki et al. 1997), and the transfection of SR-A into weakly adherent cells can confer an adhesive phenotype (Robbins & Horlick 1998). SR-A has been shown to adhere macrophages to ligands associated with specific disease conditions. Adhesion to both glucose-modified collagen IV (el Khoury et al. 1994) and advanced glycation end products (Araki et al. 1995), which may underlie the accelerated development of vascular problems in diabetics, and also to OxLDL-coated surfaces, (Maxeiner et al. 1998) occurs via SR-A. Investigations using rodent microglia and human macrophages demonstrated SR-A-mediated attachment to β-amyloid fibrils, which leads to secretion of reactive oxygen species and immobilization of the cells with possible functioning in the pathophysiology of Alzheimer’s disease (El Khoury et al. 1996). It has been shown that the cytoplasmic amino acids proximal to the membrane are required for SR-A posttranslational processing and play a critical role in SR-A trafficking to the cell surface. These amino acids were found to be needed for SR-A-mediated adhesion, but not for ligand internalization (Kosswig et al. 2003). Recently, SR-A has also been shown to mediate adhesion to proteoglycans (biglycan and decorin).
of the extracellular matrix that are present in atherosclerotic lesions (Santiago-Garcia et al. 2003).

Role in atherosclerosis

Scavenger receptors are also implicated in lipoprotein metabolism. SRs are not downregulated by high levels of intracellular cholesterol, which results in lipid accumulation and the conversion of macrophages into foam cells characteristic of atherogenesis. The contribution of SR-A to the development of atherosclerosis was examined by crossing SR-A\(^{-/-}\) animals with mice lacking apolipoprotein E, which display a high frequency of spontaneous vascular disease (Suzuki et al. 1997). The double-mutant animals displayed a reduction of about 60% of lesions, but the residual activity confirmed the participation of other SRs. If the binding of modified LDL is blocked, it is possible that the accumulation of cholesterol ester and the subsequent formation of foam cells would not occur. That is why Yoshiizumi et al. synthesized novel derivatives of sulfatides as inhibitors for SRs. The sulfate moiety was crucial, and it was the most preferable functional group for a potent inhibitory activity (Yoshiizumi et al. 2002).

MARCO

Structure and expression

Macrophage receptor with collagenous domain (MARCO) is a relatively new member of scavenger receptor class A. It was initially cloned from mouse (Elomaa et al. 1995). MARCO is disulfide-bonded trimer with a similar structure to SR-AI. Domain I forms 49 amino acids long intracellular domain which does not contain any known endocytic motifs. It has later been found that the main form of MARCO may have a cytoplasmic domain of 20 amino acids. However, no data are available on the amino acid sequence of native MARCO (Kangas et al. 1999). Domain II forms a 24-amino acid hydrophobic transmembrane domain followed by 75 residue hydrophilic domain III containing two cysteine residues and two putative N-glycosylation sites. These cysteine residues are involved in the linkage of the three chains through interchain disulfide bonds. Domain IV has 270 amino acids and it differs substantially from the coiled-coil region of the scavenger receptor, and instead has a typical collagenous sequence characterized by 89 Gly-X-Y triplets interrupted at one location by Ala-Glu-Lys. At the end of the collagenous domain there is a sequence GQKGEKGQK, which is closely related to the
lysine-rich segment in the corresponding position of SR-AI and II. The 98 amino acid long C-terminal domain V has six cysteines making it a member of group A of the SRCR superfamily. This domain showed 48.9% sequence identity with the C-terminal domain of SR-AI (Elomaa et al. 1995).

In normal mouse MARCO expression is restricted to macrophages, but not all macrophages express MARCO. It is found to be expressed on defined subsets, which are restricted to certain tissue compartments. In spleen, expression is found on the macrophages of the marginal zone. In lymph nodes, MARCO is found on the macrophages of the medullary cords. MARCO has also been found in peritoneal macrophages (Elomaa et al. 1995; van der Laan et al. 1997; van der Laan et al. 1999).

**Role in innate immunity**

MARCO has been shown to bind Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria, but not yeast (zymosan) (Elomaa et al. 1995; Palecanda et al. 1999; Elshourbagy et al. 2000). The neutral polysaccharide Ficoll, which is avidly taken up by marginal zone macrophages, did not show any binding to the MARCO molecule. Interestingly, MARCO can bind AcLDL. The binding of these ligands could be inhibited by polyG, a known polyanionic inhibitor of scavenger receptor ligand binding. Monoclonal antibodies directed against the C-terminal cysteine-rich domain block ligand binding ability of MARCO (van der Laan et al. 1997; van der Laan et al. 1999). This suggests that the binding region of bacteria resides in the C-terminal domain and is different from SR-A. However, it does not exclude that the collagenous domain is not involved in ligand binding. Same antibodies did not have any affect on overall clearance and killing of circulating living *E. coli* and *S. aureus* in mice (van der Laan et al. 1999). This suggests that there is a redundancy in the pathways by which bacteria are cleared from the blood. However, the capturing of heat-killed bacteria by macrophages in the marginal zone of the spleen was clearly inhibited. This all suggests a role in the host defense system and homeostasis of the body.

Reactive arthritis is a rather infrequent consequence of common infections by facultative intracellular Gram-negative pathogens, such as *Salmonella*. Expression of MARCO was susceptible to modulation in the inflammatory arthritis conditions of rheumatoid arthritis and spondylarthropathy (Seta et al. 2001). MARCO is a variable to be considered as a candidate factor that might contribute to reactive arthritis. High in normal (HIN) –1 is a secreted protein highly expressed in normal human breast epithelium, but is significantly down-regulated in most breast carcinomas and
preinvasive lesions (Krop et al. 2001). Homologous protein HIN-2/UGRP1 is specifically expressed by epithelial cells of the bronchioles and probably functions as an opsonin. MARCO was identified to function as a receptor for HIN-2/UGRP1 (Bin et al. 2003). The UGRP1-MARCO ligand-receptor pair is probably involved in clearance of pathogens in the lung.

The clearance of inhaled particulate matter is primarily mediated by alveolar macrophages through the process of phagocytosis (Bowden 1987). Scavenger receptors have been implicated in binding and internalization of these unopsonized environmental particles, such as oxide particles and quartz (Kobzik 1995). Alveolar macrophages from SR-A−/− mice showed no decrease in particle binding, whereas a monoclonal antibody against MARCO was able to partially inhibit particle (TiO2, Fe2O3 and latex beads) binding (Palecanda et al. 1999). This defines a novel and immunologically important function for MARCO.

Cytoskeleton rearrangements

The presence of the SRCR domain in molecules that are involved in immunological activities and expressed on the cell surface suggests that this motif is important and may be related to cellular interactions through these molecules. In concordance with the role of CD6, MARCO may be related to positioning of macrophages in their microenvironment. Ectopic expression in vitro, in nonmyeloid cell lines, induces dramatic changes in cell shape and induces the formation of lamellipodia and long dendritic processes (Pikkarainen et al. 1999). These changes are accompanied by the rearrangement of the actin cytoskeleton. The activity was found to be dependent on SRCR domain. MARCO has also been shown to be involved in cytoskeleton organization in dendritic cells and microglia (Granucci et al. 2003). The simple expression of MARCO was sufficient to induce cytoskeleton modifications in dendritic cells. MARCO-transfected immature dendritic cells acquired a typical morphology of mature dendritic cells and did not rearrange the actin cytoskeleton following activation. MARCO-expression also decreased the phagocytic activity. This might be an attempt to slow down their propagation.

SR-CLI and II

A novel collagenous receptor with a C-type lectin domain (SR-CL) was recently identified from mouse (Nakamura et al. 2001a) and human (Nakamura et al. 2001b). Northern blot analysis revealed that the gene is abundantly expressed in various adult...
human tissues. The same receptor, termed CL-P1 (collectin placenta 1), was also cloned by others (Ohtani et al. 2001). SR-CL exists in two forms, SR-CLI and II, which differ with regard to their C-terminal regions. SR-CLI has an identical domain structure with SR-AI, except that it contains a carbohydrate recognition domain (CRD) instead of a SRCR domain. CRD of SR-CLI belongs to the C-type lectin family, which are dependent on Ca^{2+} for ligand recognition. The type II form of SR-CL lacks the carbohydrate recognition domain. Ligand-binding studies with SR-CL showed binding and phagocytosis of *E. coli*, *S. aureus* and *Saccharomyces cerevisiae*. It reacts with OxLDL, but not with AcLDL. These binding activities are inhibited by polyanionic ligands. These results indicate a role in host defense that is different from that of soluble collectins in innate immunity. To study the function of the CRD, Yoshida et al. produced a secreted form of the domain. They showed that the domain specifically interacts with Gal-type ligands, T- and Tn antigens, and SR-CL-transfected cells specifically internalize GalNAc-conjugated particles (Yoshida et al. 2003). SR-CL may thus also have important function in recognition of carbohydrate-containing ligands.
Aims of the present study

At the time of joining the research group, a novel murine macrophage receptor with a collagenous structure, MARCO, had already been identified. Analysis of mouse MARCO had indicated structural similarity to SR-A, but also that the genes have very different expression patterns. Furthermore, cells transfected with the mouse MARCO cDNA had been found to avidly bind both Gram-negative and Gram-positive bacteria, but not yeast suggesting a role for MARCO in anti-microbial host-defense mechanisms (Elomaa et al. 1995). The aim of the present study was to further examine the binding properties of MARCO and study its involvement in the innate immune system.

The specific aims were:

1. To clone the full-length human MARCO for sequence comparison with the mouse protein, and also for ligand-binding studies with transfected cells.
2. To produce and characterize antibodies against human MARCO, and study its expression in pathological states using immunohistochemistry.
3. To produce the extracellular part of MARCO in biochemically sufficient amounts for structural and functional studies.
4. To use this protein, called soluble MARCO or sMARCO, to identify novel ligands of the receptor.
Methods

The methods used in this study are described in detail in the “Materials and Methods” section of each paper. Here, the methods used are listed. The number indicates the paper in which the method is described.

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Results and discussion

Characterization of human macrophage receptor

**MARCO (I)**

The human MARCO cDNA was cloned from liver and spleen cDNA libraries. Analysis of the cDNA showed that the open reading frame encodes a polypeptide chain of 520 amino acid residues. An independent group later cloned the same cDNA (Elshourbagy et al. 2000). Furthermore hamster MARCO has also been cloned (Palecanda et al. 1999). Interestingly, hamster MARCO has a shorter collagenous domain than mouse or human MARCO. The identity of the amino acid sequence between the full-length human and murine polypeptide chains is only 68%, and between hamster and mouse or human, 77 and 65%, respectively. Interestingly, the human MARCO subunit chain differs from the mouse chain in that it does not have any cysteine residues outside the cysteine-rich domain V, whereas the mouse chain (Elomaa et al. 1995) has one cysteine residue within the intracellular domain I and two in the extracellular spacer domain III. The single cysteine in the intracellular domain of the mouse chain probably has no specific role, but the two cysteine residues in the spacer domain participate in the formation of interchain disulfide bonds (Elomaa et al. 1995). Therefore, the three subunit chains of human MARCO are bound to each other only through the collagenous triple helix, as the cysteine residues in the COOH-terminal domain V all form intrachain bonds. Similarly, hamster MARCO does not have any cysteine residues in the spacer domain (Palecanda et al. 1999). Both potential carbohydrate attachment sites in the spacer domain III and the interruption of Gly-Xaa-Yaa repeats in collagenous domain IV are conserved between the three species, indicating a functional importance for these sites.

*In situ* hybridization analyses of tissues from two newborn infants revealed expression of MARCO in macrophages of several organs, such as thymus, intestine, and
kidney, as well as in Kupffer cells of the liver. The broad pattern of expression contrasts the very restricted expression pattern of mouse MARCO. However, the human autopsy samples analyzed in the present study were obtained from infants who both had died due to sepsis. In line with these data, it has turned out that in mice the MARCO gene is induced in most tissue macrophages, when the animals are challenged by bacteria, BGC or LPS (van der Laan et al. 1997; Ito et al. 1999; van der Laan et al. 1999). This is in line with the knowledge that there are several potential binding sites for transcription factors associated with inflammation in the promoter region of the MARCO gene (Kangas et al. 1999). Together, these observations strongly associate the expression of MARCO with the response of the organism against bacterial infection. In a more recent study, analysis of MARCO expression in a normal human individual indicated highest level of expression in lung (alveolar macrophages) and liver (Palecanda et al. 1999; Bin et al. 2003). MARCO appears to be expressed also by primary human monocytes, but not by T lymphocytes (Bin et al. 2003). In hamster, MARCO is expressed in alveolar macrophages, macrophages of lymph node sinuses, and in von Kupffer cells of the liver. There is expression also in macrophages in splenic red pulp, intestinal Peyer’s patch and thymus (Palecanda et al. 1999). Recent global gene analyses have associated MARCO expression to dendritic cells too. Indeed, the MARCO gene was one of the most highly upregulated genes in splenic dendritic cells after treatment with LPS or bacteria (Granucci et al. 2003). This same study indicated a similar phenomenon in GM-CSF - treated microglial cells. In an other study, the MARCO gene was found to be expressed at high levels after pulsing bone marrow-derived dendritic cells with a tumor lysate (Grolleau et al. 2003). Thus in conclusion, it seems that the MARCO gene can be readily upregulated under conditions of bacterial infection, thus forming a back-up system through which the organism can quickly arm more cells with highly efficient phagocytosing receptors.

To study the bacteria-binding capability of human MARCO, COS cells were transiently transfected with an expression construct encoding the full-length human protein. We also transfected cells with plasmids encoding truncated forms of human MARCO either completely lacking the SRCR domain or containing short segments of it. Transfected cells were incubated with FITC-labeled *E. coli* or *S. aureus*, and the bacterial binding was detected by fluorescence microscopy. The results clearly demonstrated that human MARCO binds bacteria avidly, and that the SRCR domain is not crucial for this activity. Indeed, cells expressing the form completely lacking the SRCR domain were not found to bind bacteria. Interestingly, we found that cells
expressing a truncated form containing only 22 residues of this domain bound bacteria strongly, but cells expressing a form containing 11 residues of it did not have significant bacteria-binding activity. Consequently, we may have identified the region of MARCO that is directly involved in the bacteria binding. If this amino acid sequence, RGRAEEVYYSGT (residues 432-442), is indeed the interactive region, the two arginines might play a role in the binding activity, as it can be inhibited by poly(G), a known polyanionic inhibitor of scavenger receptors (Figure 5). These results also indicate that the bacteria-binding characteristics of MARCO and SR-A are different. This is the case since for SR-A, the ligand-binding region has been mapped to a cluster of conserved lysine residues at the end of the collagenous domain (Acton et al. 1993).

The carboxyl-terminal domain V of MARCO contains six cysteine residues that form intrachain, but not interchain, disulfide bonds. Here, the disulfide bridge pattern was elucidated using human domain V produced in the baculovirus/insect cell expression system. Domain V was expressed as a fusion protein with MMP-2 matrix metalloproteinase, where it replaced the COOH-terminal hemopexin domain of the enzyme. The results demonstrated that cysteines C1 and C5, C2 and C6, as well as C3 and C4, are connected to each other by disulfide bridges. This pattern is identical to the disulfide pattern observed in the cysteine-rich domain of SR-AI expressed in CHO cells (Resnick et al. 1996). The fact that the same pattern is obtained in two different SRCR domains expressed in two different eukaryotic systems indicates that this is the pattern of native SRCR domains in vivo. This is also supported by the fact that the proteolytic fragments obtained in the experiments of this study were homogeneous and fragments with different disulfide-bond patterns were not found. Finally, since two different SRCR domains have an identical disulfide-bond pattern, it is likely that this is a general pattern in SRCR domains containing six cysteines. In line with this statement, structural analysis of the SRCR domain of Mac-2 binding protein indicated this very same disulfide-bond pattern (Hohenester et al. 1999).

In conclusion, the results of the present study yielded the primary structure of the human macrophage MARCO receptor and provided further support for its association with bacterial binding and host defense.
Figure 5. A potential bacteria-binding region of human MARCO. Amino acid sequence RGRAEEVYYSGT (residues 432-442), is the interactive region and the two arginines may play important role in the binding of bacteria.

**Analysis of the bacteria-binding activity of MARCO (II)**

To further analyze the bacteria-binding function of MARCO, we carried out studies with cells expressing receptor variants with deletions in various regions of its extracellular domain (Figure 6). All these mutants were readily transported to the cell surface as determined by immunostaining. First, we confirmed our previous findings, and showed that the cells expressing the full-length human or mouse MARCO, as well as human mutant h-442 containing the 22 most proximal amino acid residues of domain V, bound bacteria strongly. In contrast, variant h-420, whose C-terminal residue is the last residue of the collagenous domain exhibited, as shown also in paper I, practically no bacterial binding.
**Figure 6.** (A) Schematic illustration of wild-type MARCO receptor and deletion variants expressed in CHO cells. (Top) Domain structure of wild-type human and mouse MARCO with the last amino acid residue of each domain being numbered. Locations of the six cysteine residues in domain V are depicted by vertical lines. (Bottom) Depiction of the mutant MARCO variants with single amino acid replacements generated by site-directed mutagenesis. Information on the cell surface expression and semiquantitative bacteria-binding capacity of CHO transfectants is shown on the right. ++++, strong binding; +, weak but detectable binding; (+), of the MARCO variants tested for bacterial binding, this is the least active. There are only a few bacteria-binding cells in this population.
These were somewhat unexpected findings, since the ligand-binding site for SR-AI and II has been mapped to the highly conserved lysine cluster at the C-terminal end of the collagenous domain (Acton et al. 1993; Doi et al. 1993), and a similar lysine-rich cluster is also present in the corresponding region of the collagenous domain of both mouse and human MARCO. Yet, although our studies did not indicate a crucial role for this segment of MARCO in bacterial binding, we still considered the possibility that the bacteria-binding site of MARCO in fact resides within this cluster, and the N-terminal segment of domain V is only needed to promote correct folding of the binding site. It could not be excluded that the C-terminal end of the collagenous domain does not form a stable triple helix in the absence of a ‘clamp’, such as a 22-residue segment of domain V. In order to study whether domain V is indeed the predominant bacteria-binding domain in MARCO, we generated various mouse MARCO constructs encoding proteins with large internal deletions and tested the bacteria-binding capability of cells expressing these MARCO forms. First, the last 40 and 21 Gly-X-Y triplets of the 89-triplet long collagenous domain were removed (Figure 6A), generating forms that do not contain the cluster of the basic residues. Both of these variants, D299-420 and D357-420 were found to effectively bind *E. coli*, as well as Ac-LDL, clearly demonstrating that the cluster of the basic residues at the C-terminal end of the collagenous domain of MARCO is needed neither for bacterial binding nor binding of Ac-LDL. Two other mutants with a deletion of the spacer and a major part of the collagenous domain (D79-354) or a deletion of almost the entire collagenous domain (D178-419) were also tested for bacterial binding. Cells expressing D79-354 readily bound bacteria. Cells expressing D178-419 also had significant bacteria-binding activity. This latter variant contained only the first 8 Gly-X-Y triplets, which is just enough for the assembly of the trimeric MARCO. There are two basic amino acids within the 24-residue collagenous segment of this protein, but no clusters of basic residues and, therefore, it is unlikely that the collagenous segment mediates bacterial binding. These results convincingly demonstrate the essential role of domain V for the bacteria-binding activity of MARCO, and therefore indicate the first function of the SRCR domain of scavenger receptors.

Having demonstrated that at least most of the bacteria-binding activity of h-442 is due to its domain V-segment, or more specifically, to that part of the segment, which is missing from h-431, RGRAEVYYS GT, we attempted to study the role of the two arginines of this segment in bacteria-binding in the context of the full-length MARCO.
It was assumed that they are of crucial importance for the binding activity, because bacterial binding to cells expressing h-442 can be abolished by poly(G) or poly(I), known polyanionic inhibitors of scavenger receptors. These two arginines, R-432 and R-434, which are conserved in mouse (Elomaa et al. 1995), human (Elomaa et al. 1998) and hamster (Palecanda et al. 1999), as well as the third arginine present in the domain V-segment of the human MARCO variants h-431 and h-442, R-425, were substituted by alanines, and in some cases also by glutamines (Figure 6B). However, the bacteria-binding studies could not be carried out, because none of the single mutants was expressed on the cell surface (not shown). This was demonstrated by incubating the cells on ice with polyclonal anti-domain V antibody before fixation. Interestingly, the mouse MARCO replacement mutants R424A and R433A (Figure 6B) were both expressed on the cell surface. We do not know the reason for this, but it might be due to the presence of a fourth arginine residue in this region of mouse MARCO (at position 422), and that this residue is able to compensate for the loss of an adjacent arginine. However, double mutant R424A/R433A (Figure 6B) was retained inside the cell, so its bacteria-binding activity could not be assayed.

Since the mutation analysis in the context of the full-length MARCO did not give any useful information, we then focused on analyzing the C-terminally truncated MARCO variants to, at the very least, get insight to the bacteria-binding activity of these forms. We first compared mouse forms m-441 and m-430 to the corresponding human forms. A control variant m-420 exhibited very low, barely detectable bacterial binding. Surprisingly, unlike h-431, m-430 was found to bind bacteria well. These two forms, m-430 and h-431, are about 50% identical with respect to their domain V segments (Figure 6B). The only major difference is that there is an arginine at position 422 of the mouse chain and a serine in the corresponding position of the human chain (residue 423). In order to study whether this difference is crucial for bacterial binding, we substituted Ser-423 by Arg in h-431. This single Ser-to-Arg replacement had a dramatic effect on the bacteria-binding activity of h-431. The bacteria-binding capability increased to approximately the same level as that exhibited by h-442. This new form contains a closely spaced arginine-doublet within a motif RVR. Further mutations showed that both arginines are needed for strong bacterial binding (Figure 6B). Notably, the segment that is present in the strong bacterial binder h-442, but is missing from h-431, which does not exhibit significant bacteria-binding activity, contains a similar motif, RGR. Thus, these results established that the motif RXR is responsible for the high-affinity bacterial binding in these truncated MARCO forms.
We do not know whether this is the case for the full-length MARCO, but modeling of the MARCO domain structure according to the structure of the Mac-2 binding protein (Hohenester et al. 1999) suggests that there is, in fact, a cluster of arginine residues in one region of the compact domain. This cluster is formed by the first arginines of the domain, as well as by three arginines from another segment (residues 460–468). Interestingly, this latter segment also contains an RGR motif. In light of the results of the current study, it is quite possible that this cluster of several arginine residues is involved in bacterial binding.

Taken together, this part of the thesis work has convincingly demonstrated the crucial role of domain V for bacterial binding in MARCO. This is the first function indicated for a SRCR domain of scavenger receptors. This work has also provided at least some hints about the requirements for high-affinity bacterial binding. It is worth noting here that bacteria-binding activity has recently been indicated for SRCR domains of another protein, salivary agglutinin (Bikker et al. 2002). Bikker et al. first showed that a fragment of the protein composed of 13 highly homologous SRCR domains binds bacteria, such as Streptococcus mutans. Several consensus-based peptides of the SRCR domains and segments between the domains were synthesized and tested for bacterial binding. Strikingly, only one of the peptides, which exhibited 55% homology to the segment present in h-441 but absent from h-431, was found to bind bacteria. This peptide did not include a RXR motif but a QXR motif, suggesting that both motifs can mediate bacterial binding.

**Production of recombinant soluble MARCO (III)**

Although cells transfected with a MARCO-expression plasmid bind bacteria strongly, one can always argue that perhaps binding is not directly mediated by MARCO, but is a result, e.g. of the cell shape changes induced by MARCO. Thus, in order to unambiguously demonstrate that MARCO binds bacteria, one needs to assay the bacteria-binding activity of isolated MARCO-molecules. This was one reason why we undertook the approach of producing ‘soluble MARCO’ or sMARCO, i.e. a recombinant protein composed of the extracellular part of MARCO. We first tried to produce an untagged version of sMARCO in the baculovirus/insect cell system or conventionally in 293 cells, but the expression levels were low. Furthermore, we noticed that sMARCO tended to disappear when passed over various chromatographic matrices. The assumption that MARCO probably adhered to these matrices is supported by the recent finding that MARCO is the major receptor on alveolar macrophages for
the binding of unopsonized environmental particles (Palecanda et al. 1999). Therefore, we decided to produce a tagged version of sMARCO which would probably allow the development of a simple purification protocol based on affinity chromatography. Also, instead of the normal 293 cells, we used the 293/EBNA cells which allow episomal replication of plasmids containing the EBV origin of replication. The tagged version of MARCO was generated by replacing the intracellular and transmembrane domains with the signal sequence of the mouse Ig kappa-chain and a polyhistidine tag (Figure 7). We then succeeded to purify the his-tagged sMARCO from 293/EBNA-cell conditioned medium to >95% homogeneity on the Ni-NTA resin in a single step. The yield was ~0.5 mg of purified sMARCO/liter of culture medium.

![Figure 7. Schematic illustration of wild-type MARCO and sMARCO. Secreted, soluble form of MARCO was generated by replacing the intracellular and transmembrane domains with mouse immunoglobulin κ chain leader and a polyhistidine tag. Roman numbers indicate the following MARCO domains: intracellular domain (I), transmembrane domain (II), spacer domain (III), triple-helical collagenous domain (IV) and cysteine-rich C-terminal domain, domain (V).](image)

Purified sMARCO migrated as a doublet with apparent molecular masses of 72 and 65 kDa on SDS-PAGE. To test whether the presence of the two forms is due to differential N-glycosylation, 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 showed that 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. We also tried to study glycosylation by culturing the cells in the presence of tunicamycin, an inhibitor of N-glycosylation. However, the expression level decreased dramatically, so that we could no longer detect the protein on a silver-stained gel. When analyzed under nonreducing conditions, two bands, a ~70 kDa band and a slowly migrating band with an apparent molecular weight of ~220 kDa, i.e. the expected size of a trimer, could be detected. Thus, only a fraction of the sMARCO molecules synthesized in the 293/EBNA cells appears to form...
disulfide bond–stabilized trimeric molecules, indicating that the formation of the interchain disulfide-bonds is not needed for the assembly of the triple helix.

However, it was yet not clear whether sMARCO secreted from the 293/EBNA cells had in fact assembled into a triple-helix. This was a concern particularly on the ground that collagens produced in 293 cells are often somewhat underhydroxylated (Fichard et al. 1997; Frischholz et al. 1998; Tasanen et al. 2000; Areida et al. 2001). In order to get information about the assembly state of sMARCO, the purified protein was subjected for circular dichroism analysis. Indeed, 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 (Piez & Sherman 1970). The melting curve indicated a Tm of 44 °C. This result demonstrated that sMARCO had assembled into a triple helix, and this was also confirmed by exposing the protein to limited trypsin digestion. This type of assay can be used to study the structure of collagenous proteins, because a triple-helix is very resistant to trypsin digestion.

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 nm ± 2 nm average length (Figure 8A and B). The length agrees well with the calculated length of an 89 Gly-X-Y repeats containing triple-helix. There is one interruption within the collagenous domain of MARCO, but no bend is seen in the electron microscopic images. About 50% of all molecules are in monomeric form and 50% are associated by the globes (Figure 8A 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 (Figure 8C) in which 3-7 molecules are connected by terminal globes (11 % of all species involving 55 % molecules). It is possible that the formation of these complexes reflects a real property of MARCO. Supporting this notion, we have observed that the stably transfected L-cells expressing the full-length MARCO have a tendency to aggregate if kept in suspension. One can envision that this head-to-head association could be important 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.
Figure 8. **Visualization of soluble MARCO by electron microscopy.** A, Rotary-shadowed images of sMARCO in a representative field. The average length of the molecule is 82.7 nm. The terminal domains, the spacer domain and domain V, appear as small globes. C, About 50% of the molecules associate with each other via the terminal globes, forming dimers, trimers and star-like complexes. The bar represents 100 nm. B and C, rotary-shadowed images of sMARCO-molecules at a higher magnification. The bar in B and C indicates 10 and 20 nm, respectively.

Next, to address the question whether MARCO indeed directly interacts with bacteria, we assayed the binding of heat-killed FITC-labeled *E. coli* to sMARCO-coated glass coverslips. This study demonstrated that sMARCO binds bacteria very strongly. The binding could be inhibited by poly(I), a ligand of scavenger receptors. However, as expected, a control polyanion, heparin, was without any effect. Thus, sMARCO exhibits binding characteristics typical for scavenger receptors. We further showed that both the MARCO-expressing CHO cells and sMARCO bind fluorescently-labeled living wild-type *E. coli* K12, *E. coli* strain HB101, as well as *S. typhimurium* LB5010 with high affinity. Wild-type *E. coli* K12 expresses the smooth form of LPS, whereas the strains *E. coli* HB101 and *S. typhimurium* LB5010 express the rough form (Bullas & Ryu 1983; Goldberg *et al.* 1992). 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 are not needed and may even hinder MARCO-bacterial interactions. 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 to rough strains lacking the O-side chains (Alexander & Rietschel 2001). We also found that LPS is a ligand for MARCO as it bound to MARCO-expressing L929-cells and to sMARCO. However, it is possible that LPS is the only bacterial ligand of MARCO. In this regard, SR-A has been found to bind a *Neisseria* strain lacking LPS (Gordon 2002).

Finally, we also produced domain V as a recombinant protein, and compared its bacteria-binding activity to that of sMARCO. We found 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 in order to effectively bind to bacteria. It is also possible that the collagenous domain does not only function as an assembly domain of MARCO, but also directly contributes to the high-affinity bacterial binding.

In summary, we have here produced biochemical amounts of soluble MARCO which was shown to be assembled into a triple-helix and to behave functionally as expected. This protein will certainly be a very useful tool when searching for ligands of MARCO.

**The role of marginal zone macrophages and MARCO in the retention and trafficking of marginal zone B cells (IV)**

Mice deficient in the inhibitory signaling molecule SH2-containing inositol-5-phosphatase 1 (SHIP) display pleiotropic defects in macrophages, NK cells, and lymphocytes (Helgason *et al.* 1998; Wang *et al.* 2002). A prominent feature of these mice is their splenomegaly resulting from dysregulation of myeloid proliferation. The initial finding of this study was that these mice also lack marginal zone B cells and that the MARCO-expressing marginal zone macrophages are no longer organized within the marginal zone and bordering to the MOMA-1 macrophages. Instead they are redistributed to the red pulp, whereas MOMA-1 expressing cells remain unaffected. Importantly, these phenotypes were found to be due to a primary macrophage defect, since they were also found in mice where SHIP was deleted in macrophages only.

It has been shown in B cell lines that SHIP functions as a negative regulator of cellular activation by regulating the association of the positive signaling kinase Bruton's
tyrosine kinase (Btk) with the membrane, thus raising the threshold required for stimulation (Tsukada et al. 1994). Next we checked if the myeloid proliferation and marginal zone macrophage phenotype leading to the loss of marginal zone B cells might be the result of inappropriate activation of Btk in macrophages of SHIP-deficient animals (Satterthwaite et al. 1998; Mukhopadhyay et al. 2002). Disruption of Btk in macrophages might restore normal signaling thresholds in SHIP-deficient mice. Indeed, both the normal marginal zone structure and spleen size were re-established in double knockout mice, indicating that Btk is an important target of SHIP in myeloid cells in vivo. Reversion of phenotypes in SHIP−/− mice by deletion of Btk suggests that Btk is the dominant Tec family member regulated by SHIP in these cells. The observation that other members of the family are expressed in macrophages and have been shown to be able to substitute for Btk both in vivo and in knock out mice indicates a surprising degree of specificity to the SHIP inhibitory pathway (Weil et al. 1997; Tomlinson et al. 1999; Smith et al. 2001).

These results suggested that marginal zone macrophages might be critical to the retention of marginal zone B cells. This was tested by depleting the macrophages with liposomes (Buiting et al. 1993). At a low concentration there was preferential depletion of the MARCO-positive marginal zone macrophages as opposed to the neighboring MOMA-1 macrophages. Other phagocytic cells were largely unaffected. At the same time, we could observe a specific reduction in the marginal zone B cells by both flow cytometry and histological staining. Similar phenotypes were not found in op/op mice that lack the MOMA-1-expressing metallophilic macrophages and the ER-TR9 marker. Rather, an expansion of these cells was observed, indicating that the macrophage population that is required for the marginal zone B cell preservation is the MARCO-expressing marginal zone macrophages.

Next, we addressed the question of whether MARCO itself is able to interact with the marginal zone B cells and has a role in the retention of these cells. To study whether MARCO directly binds the B cells, we used sMARCO to stain splenic populations. Three populations of cells were distinguished by flow cytometry when stained with CD21 and CD23. Indeed, maximal binding to sMARCO was observed for the marginal zone B cells (CD21 hi CD23 low), whereas the follicular B cells (CD21 low CD23 hi) displayed reduced binding. On the other hand, none of the other splenic cell populations were found to be able to bind sMARCO. Further studies indicated that the interaction of sMARCO and the marginal zone B cells could be blocked by the monoclonal antibody ED31 recognizing domain V of mouse MARCO, indicating that
domain V is important for the binding. Interestingly, when this antibody was injected into a normal mouse, the marginal zone B-cells were found to reside in the follicular zone 3 h later. Further challenging studies were carried out using rhodamine-conjugated \textit{S. aureus}. Within 30 min of injection bacteria were visualized exclusively to the marginal zone macrophages. 18 h after injection the microbes and the marginal zone macrophages were found to have comigrated into the red pulp and cells with the marginal zone B cell phenotype (CD1d\textsuperscript{high}) were mostly found in the follicular region. These results are consistent with a model in which interaction of \textit{S. aureus} with MARCO on the marginal zone macrophages results in their migration into the red pulp and the simultaneous migration of the marginal zone B cells into the follicular region as has been reported for LPS and \textit{E. coli} (Gray \textit{et al.} 1984; Lu & Cyster 2002). As mentioned above, deletion of SHIP has a similar effect on the marginal zone macrophage migration, suggesting that macrophage activation can trigger migration into the red pulp. The migratory response of the marginal zone macrophages, carrying an antigen to the red pulp, could simply be a method of clearance of particulate antigens or alternatively the marginal zone macrophages could function as antigen transporters/presenters and supporters of plasmablast formation shown to take place in the red pulp (Ho \textit{et al.} 1986; Smith \textit{et al.} 1996; Sze \textit{et al.} 2000).

In conclusion, the results suggest that direct interaction of MARCO with the marginal zone B cells is important for the retention of the B cells. The ligand of MARCO on the B cells remains to be identified. Our results also suggest that the interaction between MARCO and the B cells is disturbed upon marginal zone macrophage binding and activation by microbial pathogens. Previous studies have shown that stromal cell – marginal zone B cell interaction mediated by VCAM/ICAM and LFA-1/\(\alpha\)4\(\beta\)1 has also a role in the B cell retention. Consequently, there seems to be at least two mechanisms for the retention of the marginal zone B cells, and it is possible that these mechanisms have different functions.

\textbf{Isolation of novel peptide ligands of MARCO (V)}

We have extended our studies on soluble MARCO and utilized it in a phage-display screen to find novel ligands of the receptor. Phage-display screen is an unbiased approach, and could possibly be used to find novel ligands. However, before starting the screen, we wanted to further characterize sMARCO, and studied its binding properties using plasmon surface resonance (BIAcore) system. The purified polyhistidine-tagged sMARCO was bound onto NTA-chips. Binding to the chip was very stable and there
was no detectable dissociation. Soluble MARCO was shown to bind LPS, LTA and poly(I). We detected no binding to heparin which is in line with the knowledge that heparin is not a ligand of scavenger receptors although it is a negatively charged macromolecule. Binding of LPS was followed by rapid dissociation reflecting weak binding. LTA, a surface component of Gram-positive bacteria, bound to sMARCO with similar dissociation kinetics. This finding agrees well with the observation that MARCO binds not only Gram-negative but also Gram-positive bacteria (Elomaa et al. 1995), and that LTA is a ligand of SR-A (Greenberg et al. 1996). Of the tested compounds, poly(I) clearly bound with the highest affinity since there was very slow dissociation. We did not attempt to determine the association and dissociation constants, because all tested macromolecules are very heterogenic in nature. Another reason is that there is no way to measure active concentration of sMARCO which is important for estimation of correct binding parameters (Zeder-Lutz et al. 1999).

Although MARCO recognizes both LPS and LTA, it is possible that these molecules are not the only microbial ligands of the receptor. This notion is supported by the recent finding that SR-A binds a Neisseria-strain which lacks LPS (Peiser et al. 2002b). Furthermore, MARCO seems to have endogenous ligands, too, as indicated by the results of paper IV of this thesis work. In order to find novel ligands of MARCO, we chose to employ the phage display approach, an unbiased approach, and screened a random decapeptide library to isolate sMARCO-binding phages. A number of studies have demonstrated that the phage-display system can target functionally active sites on proteins. The phage display approach has been particularly successful in the identification of integrin-binding peptides. For example, when peptides binding to integrin α5β1 were selected, about 90 % of them had the RGD motif (Koivunen et al. 1993). This is a striking result, since integrin α5β1 recognizes fibronectin via the sequence RGD.

Microtiter wells were coated with a high concentration of sMARCO and this surface was used to select sMARCO-binding phages. Four rounds of selection were performed, using much lower sMARCO-coating concentrations after the first round. Altogether, of the 31 clones sequenced from round four, only five different sequences were obtained (Table III). The two most enriched sequences were VRWGSFAAWL, which accounted for more than 60 % of the sequences from round four, and RLNWAWWLSY, which accounted for about 15 % of the phages bound to sMARCO in round four. In addition to the VRWGSFAAWL and RLNWAWWLSY sequences, three other sequences were recovered from round four clones. One of these,
IPVKWLLRWR, was recovered from three clones, and another one, LRLQWRAWLA, from two clones. The third sequence, PVRWRWASWL, occurred in one clone. In the following experiments we focused on the two most enriched ones, particularly on the VRWGSFAAWL phage.

**Table III. Isolation of peptides binding to soluble MARCO from a linear decapeptide phage display library.** The number of clones encoding the same peptide is shown in frequency.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>VRWGSFAAWL</td>
<td>20</td>
</tr>
<tr>
<td>RLNWAWWLSY</td>
<td>5</td>
</tr>
<tr>
<td>LRLQWRAWLA</td>
<td>2</td>
</tr>
<tr>
<td>IPVKWLLRWR</td>
<td>3</td>
</tr>
<tr>
<td>PVRWRWASWL</td>
<td>1</td>
</tr>
</tbody>
</table>

First, the interaction of MARCO and VRWGSFAAWL phage was confirmed in the BIAcore analysis. This analysis indicated that the phage dissociated from sMARCO slower than LPS and LTA. A control phage was not found to bind to sMARCO. Next, we were interested in localizing the binding region on MARCO. Our work has so far indicated that domain V, the SRCR domain, is the functionally active domain in MARCO. Indeed, it has been found to be crucial for MARCOs capability to induce dendritic processes (Pikkarainen et al. 1999), as well as for the bacteria-binding activity (papers I and II). Also, the sMARCO – marginal zone B cell interaction appears to be dependent on this domain (paper IV). Therefore it was of interest to investigate whether this same domain is responsible also for the phage-binding. Thus, we tested the binding of the VRWGSFAAWL and RLNWAWWLSY phages to transient CHO transfectants expressing either the full-length mouse MARCO or the truncated form lacking the SRCR domain. Experiments were done on ice, meaning that only cell-binding was measured. We found that cells transfected with the full-length MARCO bound both phages but not a control phage. Truncated MARCO did not bind either one of the phages indicating that the binding site for the VRWGSFAAWL and RLNWAWWLSY phages indeed resides in the SRCR domain. These results were confirmed in a cell-free system. Here, we utilized recombinant domain V that we had produced previously (paper III). In this experiment, microtiter wells were coated with BSA, sMARCO, recV, or recombinant Nephrin (rNephrin), and phage-binding was measured. This assay
demonstrated that the VRWGSFAAWL phage bound avidly to recV- and sMARCO-coated surfaces and there was almost no binding to surfaces coated with rNephrin or BSA. In another experiment, we counted the number of VRWGSFAAWL phages binding to the surfaces coated with these three recombinant proteins. When comparing a plating dilution which gave only 1 or 2 colonies on the rNephrin-plate, there were ~200 colonies on the recV-plate and more than 1000 colonies on the sMARCO-plate showing that the monomeric SRCR domain clearly binds the VRWGSFAAWL phage, but the trimeric sMARCO appears to have higher binding affinity. Next, the VRWGSFAAWL peptide was produced as a GST fusion protein, and binding to the MARCO-transfectants was tested. The results were in line with the phage-binding results, and confirmed that it is the SRCR domain of MARCO that recognizes the VRWGSFAAWL peptide. There was no detectable binding of GST alone. Next, since all the assays so far had been carried out with mouse MARCO, we were interested in studying whether the binding is specific for the mouse protein, or if MARCO from another species can also bind the peptide. Therefore, we carried out binding assays with the human MARCO transfectants. Indeed, these cells were found to bind the VRWGSFAAWL peptide equally strongly as the mouse MARCO transfectants.

In conclusion, we have identified two novel peptides that bind to the SRCR domain of MARCO. These results seem to strengthen the notion that the SRCR domain is the ligand-binding domain in MARCO. It remains to be seen whether the peptides recognize the same site in the SRCR domain. However, they appear to have common features, i.e. an N-terminal basic residues followed by a stretch of mostly hydrophobic residues. In this regard, it is worth mentioning that we also prepared the VRWGSFAAWL peptide in a synthetic form, but it was poorly soluble in aqueous solutions, and therefore its activities could not be assayed reliably. However, the GST-VRWGSFAAWL peptide fusion protein was not found to block the cell shape changes induced by MARCO, or its bacteria-binding activity, and therefore these experiments did not give an answer to the question of whether the peptide represent a bacterial ligand or a ligand in serum.

Finally, the VRWGSFAAWL and RLNWAWWLSY phage sequences were analyzed using the BLAST program (NCBI) to search for similarities with known or hypothetical proteins deposited in the databank. However, since it is possible that the selected phage sequences do not represent linear sequences present in ligand(s), but rather a structural mimetope, the BLAST search may not identify all potential ligands. That is why we decided to raise polyclonal antibodies against the VRWGSFAAWL
peptide, and use them in the search of the ligand(s) of MARCO. The function of antiserum was tested in ELISA and Western blot, and was found to be specific against VRWGSFAAWL peptide. Reactive immunoglobulins were purified against the immobilized fusion protein and we used these antibodies to probe mouse liver and spleen extracts, mouse serum and E. coli extract. Interestingly, the antibodies detect a similar doublet of ~ 85 – 90 kDa in the liver and spleen extracts, but not any specific bands in the mouse serum. Furthermore, a very strong signal of the size ~ 60 kDa was detected in the E. coli extract. It is very possible that these immunoreactive bands represent novel ligands of MARCO.
Concluding remarks

This thesis work started at the end of 1995, at the time when Outi Elomaa and her colleagues had just cloned a novel mouse SR-A-like scavenger receptor with an interesting expression pattern. Ever since, we have been pursuing this work to explore the role of MARCO for immune defense. Why else would nature had created a SR-A-like molecule as SR-A itself is already expressed in almost all macrophage populations?

We believe that this work has provided several contributions to the scavenger receptor research. First, we cloned the full-length human MARCO, which provided an important tool for transfection studies. Expression analysis of human MARCO gave seemingly contrasting results to those obtained with mouse MARCO. However, the human individuals analyzed had died because of a bacterial infection, and the mouse MARCO gene was also found to be induced in many macrophage populations during an infection. More recent analysis of the hamster and human MARCO expression has indicated expression, for example, in alveolar macrophages (Palecanda et al. 1999; Bin et al. 2003). Therefore, it is possible that the very restricted expression pattern of MARCO in normal mice is due to the sterile and clean living conditions, even if strain specific differences may also play some role (Su et al. 2001).

We put extensive efforts into localizing the region on MARCO important for bacterial binding. These studies clearly demonstrated that the C-terminal domain, the SRCR domain/domain V, is the predominant bacteria-binding domain in MARCO. This is an important finding since the corresponding domain of SR-A is still without any known function. And altogether, very little is known about the functions of SRCR domains in different proteins. We also used a whole series of C-terminally truncated forms of MARCO to understand the binding requirements. We could identify a short motif, RXR, responsible for high-affinity binding – at least in case of these trimeric MARCO truncations. It remains to been seen whether the same is true for intact MARCO. However, it is quite likely that only co-crystallization of MARCO, or its...
SRCR domain, with a bacterial ligand will allow unambiguous localization of the binding site.

Probably the most important contribution of this thesis work to the scavenger receptor research is the establishment of the production and purification system for recombinant soluble MARCO. It took a long time and efforts to reach this goal. We finally succeeded by using the 293/EBNA system which allows for episomal replication of the expression plasmid. Structural characterization of the purified protein already provided putatively interesting information about the properties of MARCO: a major portion of the protein was associated with itself via the terminal globes. This observation possibly reflects a real property of the protein.

Soluble MARCO has already been used in the last two parts of this thesis study. In one of those, sMARCO was crucial for the demonstration of MARCO – marginal zone B cell interaction. The demonstration of this interaction is an important finding because it indicates that there are also endogenous ligands for this scavenger receptor. In the other work, we took a different approach, and searched short MARCO-binding peptides from a complex, random peptide library. This work resulted in the selection of only a handful of peptides. The work is currently in progress to find the protein(s) that these peptides represent. Yet, we already know that it is the SRCR domain where these peptides bind in MARCO. These results have therefore strengthened our notion, that the SRCR domains constitute the biologically active part of MARCO. Maybe the long collagenous domain is just a stalk, which is needed only for the assembly of the subunit chains and extension of the active domain far above the lipid bilayer.
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