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Structure and Biological Properties of Scavenger Receptor MARCO

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Stockholm 2002

Doctoral thesis:
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ISBN 91-7349-294-9

Published and printed by Karolinska University Press
Box 200, SE-171 77 Stockholm, Sweden

To my family

SUMMARY

Macrophages are monocyte-derived cells that play an important role in the innate immune response against invading pathogens. These cells express several host defense receptors that can be divided into two classes; those dependent on opsonizing components for recognition of pathogens, and those that can recognize pathogens directly, pattern recognition receptors (PRRs).

Class A scavenger receptors are a family of PRRs composed of three members: Scavenger Receptor A (SR-A), MAcrophage Receptor with COllagenous structure (MARCO), and a recently identified protein Scavenger Receptor with C-type Lectin (SRCL). MARCO is a trimeric membrane protein containing an N-terminal intracellular domain, a transmembrane domain, and an extracellular portion composed of a short spacer domain, a triple-helical collagenous domain, and a C-terminal cysteine-rich domain (SRCR). In unstimulated mice, MARCO expression is restricted to the marginal zone macrophages in spleen, macrophages in medullary cord in lymph nodes, and peritoneal macrophages. Its expression can be induced in cultured macrophages or in macrophages of various tissues after administering LPS, BCG, *Listeria monocytogenes* or zymosan. These findings suggest a role in host defense. Furthermore, cells transfected with MARCO cDNA avidly bind both gram-negative and gram-positive bacteria, but not yeast. Preliminary data shows that the bacteria-binding region is in the SRCR domain. Interestingly, MARCO was found to be the major receptor on alveolar macrophages for binding of unopsonized environmental particles such as TiO_2 and Fe_2O_3.

To examine the regulatory mechanisms of MARCO, and its potential role in disease, the structures of human and murine MARCO genes were determined. Both genes have 17 exons, of which exons 4-15 encode the collagenous domain. Two major transcription initiation sites were identified, one starting at position +1, 27 bp downstream of a TATA box, and another at position –64, downstream of an AT-rich region. Several potential binding sites for transcription factors involved in host defense were identified in the promoter region. The human and mouse genes were localized to syntenic regions on chromosomes 2 and 1, respectively.

We could also show that MARCO most likely has a direct effect on the phenotype of activated macrophages, since the expression of MARCO in different cell lines induces dramatic cell shape changes. Typically these changes include formation of large lamellipodia-like structures and long dendritic processes. The morphological changes are accompanied by disassembly of actin stress fibers and often also by complete loss of focal adhesions. The MARCO-induced changes are dependent on cell adhesion and are inhibited when the cells are plated on fibronectin-coated surfaces. Similarly, a dominant-negative mutant of Rac-1 partially inhibits the morphogenic effects of MARCO in CHO cells. Our data indicate that the proximal segment of the SRCR domain is important for the morphoregulatory activity.

We used a large number of human and mouse MARCO variants to show that the predominant bacteria-binding region of MARCO resides in the SRCR domain. This result demonstrates the first function for a SRCR domain of the scavenger receptor A family. In further analysis, we found that an arginine-rich segment in the cysteine-rich domain is responsible for the high-affinity binding. More precisely, the motif arginine-X-arginine was found to be of importance in this regard. This was demonstrated by comparing the bacteria-binding activity of two truncated constructs of MARCO. One form, which contains an arginine-doublet in its SRCR domain, binds bacteria avidly, while the other, containing one single arginine residue, exhibits insignificant binding.

To obtain a tool for ligand-binding studies, we produced the extracellular part of MARCO as a recombinant protein, sMARCO. Several studies indicated that sMARCO has a triple-helical structure. When imaged in electron microscope after rotary shadowing, the protein appeared as 82.7 nm long rod-like molecules with small globes at both ends. Furthermore, the molecules were often found to be associated with each other. Binding studies with sMARCO, as well as with MARCO-expressing cells, indicated that MARCO binds gram-negative strains expressing either the smooth- or rough- form of LPS. Additional studies revealed that LPS is a ligand of MARCO. Finally, we also produced the SRCR domain as a recombinant protein, and compared the bacteria-binding properties of this protein and sMARCO. These studies indicated that the SRCR domain has to be in a trimeric form in order to bind bacteria efficiently.

CONTENTS

ABBREVIATIONS

AcLDL	Acetylated low-density lipoprotein
ALCAM	Activated leukocyte cell adhesion molecule
AM	Alveolar macrophage
APC	Antigen-presenting cells
BCG	Bacillus Calmette-Guerin
BPI	Bacterial permeability increasing protein
BSA	Bovine serum albumin
CD	Circular dichroism
CRP	C-reactive protein
ECM	Extracellular matrix
FCS	Fetal calf serum
FISH	Fluorescence *in situ* hybridization
IFN	Interferon
IL	Interleukin
LBP	LPS-binding protein
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MALT	Mucosal-associated lymphoid tissue
MARCO	Macrophage receptor with collagenous structure
MBL	Mannose-binding lectin
M-CSF	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex
OxLDL	Oxidized low density lipoprotein
PALS	Periarteriolar lymphoid sheath
PAMP	Pathogen-associated molecular pattern
PBM	Peripheral blood monocytes
PGRP	Peptidoglycan recognition protein
Poly(A)	Polyadenylic acid
Poly(C)	Polycytidylic acid
Poly(G)	Polyguanosinic acid
Poly(I)	Polyinosinic acid
PRR	Pattern-recognition receptor
SP	Surfactant protein
SR	Scavenger receptor
SRCL	Scavenger receptor with C-type lectin
SRCR	Scavenger receptor cysteine-rich domain
TGF	Transforming growth factor
TIR	Toll/IL-1 receptor domain
TLR	Toll-like receptor
TNF	Tumor necrosis factor

PREFACE

Lymphoid organs, such as spleen and lymph nodes, are important sites of our immune system. It is at these sites where antigens are eliminated from the blood or lymph by macrophages, or where they meet their specific T- or B-cell receptors and trigger the adaptive immune responses. However, the adaptive immune responses are not the only mechanisms used by the body to combate invading pathogens. The adaptive immunity works together with an ancient system known as innate immunity. This latter form of immunity exists in all multicellular organisms and functions as the front line against infections. One of the most important cells in the innate immunity are the macrophages. The first interaction between a pathogen and the macrophage is mediated by the germ-line encoded receptors, i.e. the pattern-recognition receptors (PRRs). They bind to invariant structures shared by a large group of microorganisms. In this work we have studied one of the macrophage PRRs called MARCO. MARCO is expressed on macrophages in spleen, lymph nodes,and peritoneum in mice living in sterile conditions, but it is markedly upregulated on macrophages in many tissues during infection.We have elucidated the structure and chromosomal localizations of both mouse and human MARCO genes, as well as studied the properties of MARCO, such as morphological features and bacteria-binding residues. We have also the produced and characterized a recombinant form of MARCO (sMARCO). Our studies indicate that MARCO may have multiple activities that contribute to the clearance of invading pathogens.

The single factors involved in innate immune system are difficult to study since the inherited defects are rare. In addition, many knockout mice missing one factor have to be challenged in the “right” way to discover a function of the deleted gene product. Recently, attempts to study the innate immune system in invertebrates have become fruitful, since their survival from pathogens relies totally on innate immune functions. There are no known invertebrate homologues to the MARCO receptor and our chromosomal localization of the human MARCO gene did not reveal any connection to a human disease. Consequently, the question what impact MARCO has in innate immunity still awaits the results from infection studies with MARCO knockout mice. The “Review

of Literature" section focuses mostly on what is known about the functions of pattern recognition receptors, especially MARCO and the structurally closely related scavenger receptor A (SR-A). Moreover, the function of macrophages, particularly those in spleen and lymph nodes where MARCO is constitutively expressed, are reviewed.

LIST OF PUBLICATIONS

This thesis is based on the following publications, which will be referred to in the text by their Roman numerals:

I. Kangas M.*, Brännström A.*, Elomaa O., Matsuda Y., Eddy R., Shows T. B., and Tryggvason K. Structure and Chromosomal Localization of the Human and Murine Genes for the Macrophage MARCO Receptor. *Genomics.* 1999; 58, 82-89.
* These authors made equal contributions to this work.

II. Pikkarainen T., Brännström A., and Tryggvason K. Expression of Macrophage MARCO Receptor Induces Formation of Dendritic Plasma Membrane Processes. *The Journal of Biological Chemistry.* 1999; 274, 10975-82.

III. Brännström A., Sankala M., Tryggvason K., and Pikkarainen T. Arginine Residues in Domain V Have a Central Role for Bacteria-Binding Activity of Macrophage Scavenger Receptor MARCO. *Biochemical and Biophysical Research Communications.* 2002; 290, 1462-69.

IV. Sankala M., Brännström A., Schulthess T., Bergmann U., Morgunova E., Engel J. Tryggvason K., and Pikkarainen T. Characterization of Recombinant Soluble Macrophage Scavenger Receptor MARCO. *The Journal of Biological Chemistry.* (In press).

REVIEW OF THE LITERATURE

Immune system

In the 19th century Robert Koch proved that infectious diseases are caused by microorganisms. There are four broad categories of disease-causing microorganisms, or pathogens: these are viruses, bacteria, pathogenic fungi, and parasites. Immunity refers to the ability to resist disease-producing microorganisms, poisons and foreign proteins. Two general systems of immunity to infectious agents have been selected during evolution: innate, or natural immunity, and adaptive, or specific immunity. The former is present in all multicellular organisms, whereas the latter evolved 400 million years ago, and is found only in vertebrates. The innate immunity, including e. g. phagocytes, natural killer cells, anti-microbial peptides, and complement factors, is activated when pathogens are encountered for the first time without previous exposures. The adaptive immunity involves activation of B- and T-cells resulting in production of antibodies and immunological memory. A fundamental difference between innate and adaptive immunity is the means by which they recognize microorganisms. Innate immune recognition is mediated by a relatively small set of structurally diverse germ-line encoded receptors, while the adaptive immune system is mediated by the highly specific antigen receptors of B-cells (immunoglobulins) and T-cells (T-cell receptors), which are derived by clonal selection (non-germ-line encoded).

Lymphatic organs

Lymphoid organs are divided into primary and secondary lymphoid organs. The primary organs include bone marrow (B-cells) and thymus (T-cells), where the development of lymphocytes occur. The secondary organs are; lymph nodes, which collect antigens from the tissues; spleen, which collects antigens from the blood; and mucosal-associated lymphoid tissue (MALT), which collects antigens from epithelial surfaces of the body, e.

g. intestine and lung. They are important for bringing antigens together with their specific T- and B-cell receptors and activate the adaptive immune system.

Lymph nodes

The lymph nodes are highly organized lymphoid structures that collect extracellular fluid from tissues, filter it from pathogens, and return it to the blood. The afferent lymphatic vessels also carry antigen-presenting cells (APCs), mostly dendritic cells, from the sites of infection in most part of the body to lymph nodes. The lymph enters the node by afferent lymphatic vessels, into the marginal sinus, from where it diffuses into cortex and paracortex areas, which consist of B-cells and T-cells, respectively. Finally it passes into the medullary sinus, where it is collected and where it leaves the node. The medullary sinus consists of strings known as the medullary cords. These include macrophages, which function to clear the lymph by phagocytosis, and antibody secreting plasma B-cells.

Spleen

The adult spleen contains the largest collection of lymphatic tissue in the body. It performs the same functions for the blood as the lymph nodes perform for the lymph. In summary, the functions of the spleen are degradation of red blood cells, phagocytosis of abnormal blood components, and initiation of appropriate immune responses by B- and T-cells. The cellular components constitute the pulp of the spleen. The red pulp consists of mostly red blood cells, whereas the cells of the white pulp resemble lymphocytes. The white pulp is divided into periarteriolar lymphoid sheath (PALS), containing mostly T-cells, and the B-cell corona. Between the white and red pulp is the marginal zone. The splenic artery branches extensively when entering the spleen, and the fine branches are surrounded by the white pulp . The lymphocytes are captured for hours, but the plasma and red blood cells are recollected in efferent lymphatic vessels, which reach the marginal zone and the red pulp. The marginal zone of the spleen (reviewed by Kraal, 1992) has a sinus, which is a cleft-like space surrounding the white pulp into which a part of the arterial blood stream opens, and where many white pulp capillaries terminate. This

provides an ideal environment for the marginal zone to function as an area where the filtration and phagocytosis from the blood occurs.

Marginal zone macrophages

There are two major macrophage subpopulations in the marginal zone, metallophilic and marginal zone macrophages. The metallophilic macrophages have low phagocytic activity, and express a receptor for sialic acid-containing glycoconjugates (Kraal, 1992). They stain positively for the monoclonal antibody MOMA-1, which has an unknown determinant (Kraal and Janse, 1986).

Marginal zone macrophages express MARCO (Elomaa *et al.*, 1995) and stain positive for ERTR-9, the antigen of which is not known (Dijkstra *et al.*, 1985). Marginal zone macrophages are large cells with long processes and appear to have close contact with the surrounding marginal zone B-cells. They do not express MHC class II antigens. They are highly phagocytic cells which can take up material, even without opsonization. For example, they have been shown to take up thymus-independent type 2 antigens. These are e. g. bacterial polysaccharides, such as the polysaccharide capsule surrounding many common extracellular pathogens, as well as neutral polysaccharides including dextran and Ficoll (Kraal, 1992).

Innate immunity

The innate immune system is the frontline against infections in the body , if not counting the physical barriers such as skin or mucosal surfaces. Since there is a delay in 4-7 days before the adaptive immune system is fully activated, the innate immune system has a very important task to protect the body in the meantime. It can also often deplete the infection without involvement of the adaptive immune system. There are known inherited defects in the innate immune system, e. g. unfunctional complement factors, but they are rare. Therefore it has been difficult to study the function of single factors in innate immunity. One way to study this ancient system is to work with invertebrates, whose survival from pathogens relies totally on the innate immune system that is strikingly similar to that in vertebrates. Recently, the discovery of Toll receptors has shed new light

on the function of the innate immune system, and has provided evidence for a connection between the adaptive and innate immune system. This is an indication that both systems are absolutely necessary for the function of immune defense in vertebrates. The innate immune system can instruct the adaptive immune system about the nature of the pathogenic challenge in many ways. Dendritic cells, which are the most efficient APCs when activated, need to be activated to efficiently present antigens to T-cells. This activation of dendritic cells are dependent on cytokines such as TNF-α and IL-1, which are secreted by macrophages in response to microbial products. In addition, effector cells in innate immunity produce different cytokines depending on what kind of T-cell response will be the most effective to a certain pathogen. IL-12 and IFN-γ cause the development of T-helper 1 cells, and IL-4 and IL-13 drive the differentiation of T-helper 2 cells. Another way for the innate immune system to influence the adaptive immunity is via complement factor C3b, which binds to the surface of the microbe. The complement receptors on B-cells and follicular dendritic cells now have the opportunity to bind the pathogen efficiently, and activate the humoral adaptive response.

Macrophages

All cellular elements of the blood, including red blood cells, platelets, and white blood cells, derive ultimately from the same progenitor or precursor cells - the hematopoietic stem cells in the bone marrow. Initially, they give rise to stem cells of more limited potential. The myeloid progenitor is the precursor of the granulocytes, macrophages, dendritic cells, and mast cells. Macrophages are one of the three types of phagocytes in the immune system, the others are dendritic cells and neutrophils. The term "macrophage" was first used by Metchnikoff more over hundred years ago to describe the phagocytic cells observed in tissues. They are the mature form of monocytes, which circulate in the blood and differentiate continuously into macrophages upon migration into tissues. Then, they are transformed into specific tissue macrophages, e. g. osteoclasts in bone, Kuppfer cells in liver, microglia in brain, and alveolar macrophages in lung. Most of the macrophages in adult tissues are derived from blood monocytes, but there is also a small local production of macrophages in sites such as spleen and lung. In tissues,

the mature resident macrophages have a relatively long life span of weeks rather than days.

Macrophages have multiple functions in the body. They participate in the overall maintenance of tissues, e. g. in bone the osteoclasts are responsible for resorbing bone, and in spleen macrophages are responsible for the recognition and elimination of senescent blood cells. Macrophages also remove dead cells and debris in repairment after injury or in tissue remodelling during growth and ontogeny. In the bone marrow macrophages play a nurturing role during maturation of red and white blood cells. In addition, macrophages play an important role in the innate immunity, since they are in the first line of defense against invading pathogens.

The first interaction between the pathogen and the macrophage is mediated by germ-line encoded receptors. They can be divided into two classes; those dependent on opsonizing components for recognizing pathogens (i. e. Fc receptors and complement receptors), and those that can recognize pathogens directly, i. e. the pattern recognition receptors (PRRs). Detection of a foreign substance in the body activates macrophages, and leads to the release of chemokines and cytokines, which results in recruitment and activation of more leukocytes. Macrophages can also act as a link between the innate and adaptive immunity in at least two ways. First, the pattern of cytokines and/or chemokines produced by activated macrophages plays a crucial role in selection of which major form of adaptive immunity is selected to predominate in response to the specific microorganism. Second, they can act as APCs. Moreover, macrophages are able to ingest pathogens via phagocytosis, and destroy them without the help of the adaptive immune system.

There are mouse strains that serve as models for macrophage deficiency. For example, op/op mice are an animal model for osteopetrosis (marble bone disease). These mice are used to investigate the differentiation mechanism of macrophage subpopulations in the absence of functional macrophage colony-stimulating factor (M-CSF). The mice are severely monocytopenic. Osteoclasts, metallophilic and some subpopulations of marginal zone macrophages in spleen are absent, while other tissue macrophages are not affected, suggesting different dependency on M-CSF among macrophage subpopulations (reviewed by Naito *et al*., 1997). Another interesting model when it comes to macrophage

deficiency are the mice with a targeted disruption in the PU.1 DNA binding domain (McKercher *et al*., 1996). They are born alive but die of severe septicemia within 48 hours. Mice maintained on antibiotics survived up to 17 days, and developed normal appearing T-cells but no mature B-cells or macrophages.

Pattern recognition receptors

PRRs are germ-line encoded receptors that bind to non-opsonized pathogens. They recognize invariant structures shared by a large group of microorganisms, pathogen-associated molecular patterns (PAMPs). These include lipoteichoic acid (LTA) and lipopolysaccharide (LPS) that are common components of gram-positive and gram-negative bacteria, respectively; double-stranded RNA that is a structural signature of several groups of RNA viruses; and mannans that are conserved components of yeast cell walls. Functionally, PRRs can be divided into three types: humoral proteins circulating in the plasma, endocytic receptors expressed on cell surface, and signalling receptors that can be expressed either on the cell surface or intracellularly. Among the soluble PRRs are soluble CD14, LPS-binding protein (LBP), bacterial permeability increasing protein (BPI), lysozyme, C-reactive protein (CRP), defense collagens (C1q, mannose binding lectin (MBL), pulmonary surfactant proteins (SP-A and SP-D)), and complement. They function by flagging microbial cells for being phagocytosed or for their destruction by complement (Uthaisangsook *et al*., 2002). Cellular PRRs are expressed on effector cells of the innate immune system, such as macrophages and dendritic cells, as well as on cells that are the first to encounter pathogens during infection, such as surface epithelia. CD14, mannose receptor, complement receptors, and scavenger receptors are all cell surface PRRs. The only known signalling PRRs expressed on cell surface are the Toll receptor family (Kopp and Medzhitov, 1999). Several PRRs are expressed in the cytosol where they can detect intracellular pathogens and induce responses that block their replication. An example is the protein kinase PKR, which binds dsRNA produced during some viral infections. Activated PKR phosphorylates and inactivates the translation initiation factor elF2a, which results in the block of viral and cellular protein synthesis (Clemens, 1997).

Table I: PAMPs and pattern recognition receptors (molecules)

PAMP	Pathogen(s)	Pattern Recognition Receptors (Molecules)
LPS	Most gram-negative bacteria	LBP, BPI, CD14, TLR4, SR-A, MARCO*
Atypical LPS	*Leptospira* *Porphyromonas*	TLR2
Lipoproteins	Many bacteria	TLR2
Peptidoglycan	Most bacteria	CD14, TLR2, lysozyme
LTA	Many gram-positive bacteria	TLR2, TLR4, SR-A
Unmethylated CpG DNA	Many microbial pathogens	TLR9
Phosphorylcholine	Many microbial pathogens	CRP
Lipoarabinomannan	Mycobacteria	TLR2
Mannans and mannoproteins	Yeast	Mannose receptor, MBL
Zymosan (yeast cell wall)	Yeast	Mannose- and β-glucan-receptors, TLR2
Carbohydrate moieties on microbial surfaces		MBL, SP-A, SP-D
Flagellin	Many bacteria	TLR5
dsRNA	Most viruses	TLR3, PKR

* Showed in paper IV in this thesis. Combined from Aderem & Ulevitch 2000, Uthaisangsook *et al.* 2002, and Medzhitov 2001.

Mannose receptor

The mannose receptor is expressed on macrophages, dendritic cells, as well as on lymphatic and sinusoidal endothelium. It has been implicated in binding and phagocytosis of a wide range of microorganisms such as bacteria, fungi and protozoa (Stahl and Ezekowitz, 1998). The mannose receptor seems to have an important role in the cytokine production when macrophages are phagocytosing chitin particles and mannan-coated beads (Shibata *et al.*, 1997). Studies on mannose receptor-deficient mice have shown that the mannose receptor is essential for the phagocytosis and clearance of inflammatory glycoproteins and regulating the serum glycoprotein homeostasis in mice (Lee *et al.*, 2002).

Toll receptors

The first member of the Toll family, *drosophila* Toll, was discovered as a necessary factor in the embryonic development of dorso-ventral axis (Belvin and Anderson, 1996). The Toll receptor family now includes at least nine receptors in *Drosophila*, and 10 mammalian Toll like receptors (TLRs) (Uthaisangsook *et al*., 2002).The *Drosophila* Toll mutants have striking defects in the immune defense against gram-positive bacteria (Khush *et al*., 2001) and fungi, but the response to gram-negative bacteria appear normal (Lemaitre *et al*., 1996). Interestingly, *drosophila* Toll does not function directly as a PRR, but is activated by gram-positive bacteria through a circulating Peptidoglycan recognition protein, PGRP-SA (Michel *et al*., 2001). The defense against gram-negative bacteria in *Drosophila* is dependent on a different pathway, known as the Immune deficiency (IMD) pathway. It involves a PRR called Peptidoglycan recognition protein LC (PGRP-LC) (Gottar *et al*., 2002; Ramet *et al*., 2002).

Mammalian Toll receptors are expressed in a variety of cell types, most predominantly in cells of the immune system, such as APCs. They have been reported to be involved in innate immunity as signalling PRRs, and in controlling the maturation of dendritic cells and the differentiation of T-helper cells. It was first shown by Medzhitov and coworkers (Medzhitov *et al*., 1997), who demonstrated that a constitutively active mutant of TLR4 induces expression of cytokines and co-stimulatory molecules on APCs. Several evidences indicate that TLR4 functions as a signalling receptor in response to LPS (see "*LPS and LPS-binding proteins"*). It is also implicated in the recognition of lipoteichoic acid (LTA), the heat-shock protein hsp60, and the fusion protein of the respiratory syncytial virus (Kurt-Jones *et al*., 2000; Ohashi *et al*., 2000; Takeuchi *et al*., 1999; Vabulas *et al*., 2001). TLR2 recognizes the largest number of ligands, such as peptidoglycan from gram-positive bacteria (Takeuchi *et al*., 1999), bacterial lipoproteins (Brightbill *et al*., 1999), and zymosan (Underhill *et al*., 1999). In addition, TLR2 functions as a receptor for atypical LPS, which are structurally different from gram-negative LPS (Werts *et al*., 2001). This unusually broad range of ligands recognized by TLR2 is explained, in part, by cooperation between TLR2 and at least to other TLRs:

TLR1 and TLR6 (Ozinsky *et al.*, 2000) (see figure 1). For more TLRs and their ligands see table I.

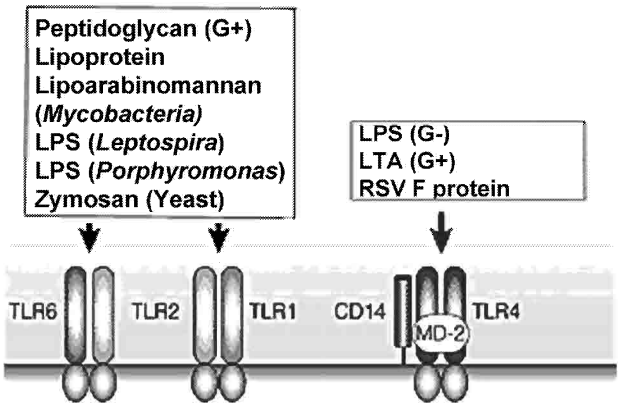

Figure 1. Ligand specificities of some of the TLRs. TLRs recognize a variety of PAMPs. Recognition of LPS by TLR4 is aided by two accessory proteins: CD14 and MD2. TLR2 recognizes a broad range of structurally unrelated ligands and functions in combination with several (but not all) other TLRs, including TLR1 and TLR6. G+, gram-positive; G-, gram-negative; GPI, glycophosphoinositol; RSV, respiratory syncytical virus. Adapted from Medzhitov 2001.

The common signaling pathway for the mammalian Toll receptors leads to activation of NFκB, which results in the induction of a variety of effector genes. It seems to be shared by all members of Toll and IL-1 receptor (IL-1R) families, and includes four essential components: the adaptor proteins, MyD88 and TOLLIP (Toll-interacting protein); a protein kinase, IRAK (IL-1R-associated kinase; and another adaptor protein, TRAF6 (TNF-receptor associated factor 6) (reviewed by Medzhitov, 2001).

LPS and LPS-binding proteins

Bacterial lipopolysaccharides (LPS) are surface structures of most gram-negative bacteria. LPS acts as a potent activator of the innate immune system in species from insects to man. LPS are heat-stable molecules composed of a lipophilic region, lipid A, and a hydrophilic polysaccharide portion. The polysaccharide portion is divided into the core, which is next to lipid A part, and the outermost O-specific chain (see figure 2).

Mutant strains without the O-specific chain are referred to as rough strains, while the wild-type strains are called smooth strains. In culture, both kinds of strains survive readily, but in tissues and body fluids most of the pathogenic bacteria need the O-specific chain to survive the attack from phagocytes and serum complement. Lipid A and the inner core structure form an extremely negatively charged region, which has been found to be the target for positively charged host defense peptides and proteins, such as the family of the mammalian defensins. The conserved structure of lipid A is recognized by the innate immune system as a PAMP, and has been proved to be the common immunostimulatory part of LPS (Alexander and Rietschel, 2001).

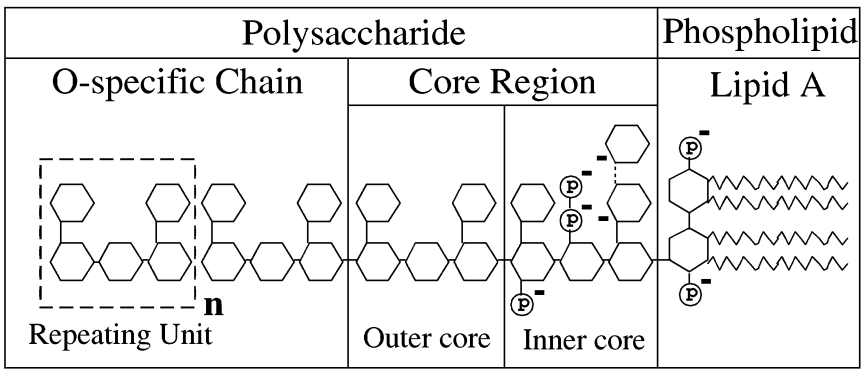

Figure 2. General chemical structure of LPS. All forms of LPS consist of the membrane-anchoring lipid A domain and a covalently linked polysaccharide portion. The terminal O-specific chain is formed of repeating units. The inner and the outer core are commonly distinguished. Adapted from Alexander and Rietschel 2001.

A severe infection of gram-negative bacteria can cause a condition known as sepsis or endotoxic shock. It is characterized by the release of pro-, and anti-inflammatory mediators secreted mainly by myeloid inflammatory cells. Severe sepsis is a life-threatening condition, including symptoms such as high fever or dramatic acute hypotension followed by multi-organ dysfunction or failure. The primary target cells for LPS in mammalian species are the professional phagocytes, including monocytes, tissue macrophages, neutrophils, and dendritic cells. Neutrophils constitutively express CD14 and TLR4, while dendritic cells are TLR4-positive cells. According to current state of knowledge, phagocyte activation by LPS or lipid A is initiated by the combined actions

of LBP, the membrane-bound or soluble form of CD14, and the TLR4*MD-2 complex on the cell surface. This leads to intracellular signaling, such as activation of MAP kinase cascades and translocation of NFkB (Alexander and Rietschel, 2001).

Studies in mice have shown that CD14 and TLR4*MD-2 complex are critical components in the response to LPS (see figure 1). Transgenic mice expressing human CD14 are hypersensitive to LPS (Ferrero *et al.*, 1993). Conversely, CD14 -/- mice are 100-fold less sensitive to LPS and *E. coli* compared to wild-type control animals. CD14-deficient mice also show resistance to bacterial spread (Haziot *et al.*, 1996). Positional cloning analysis of the LPS-nonresponsive mouse strain C3H/HeJ demonstrated that a point mutation in the Toll/IL-1 receptor (TIR) domain of TLR4 is responsible for the defect in LPS signal transduction. The TIR domain is the intracellular domain of the mammalian Toll receptors. An additional mouse strain, B10.ScCR, also turned out to be hyporesponsive to LPS. Further investigation showed that B10.ScCR mice lack the entire *tlr4* gene (Poltorak *et al.*, 1998; Qureshi *et al.*, 1999). Mice with a targeted deletion of the *tlr4* gene are unresponsive to LPS as well (Hoshino *et al.*, 1999). Recently, Nagai *et al* (Nagai *et al.*, 2002) showed that MD-2 knockout mice did not respond to intraperitoneal administration of LPS, and therefore survived endotoxic shock. In addition, MD-2-deficient mice were reported to be more susceptible to *Salmonella typhimurium* compared to the control animals. These phenotypes are identical to TLR4 -/- mice, demonstrating the absolute requirement for MD-2 in TLR4-dependent LPS responses. It turned out that TLR4 was not able to reach the cell membrane in MD-2 -/- cells, indicating that MD-2 is essential for the intracellular distribution of TLR4.

Scavenger receptors

The scavenger receptor (SR) family consists of a both functionally and structurally diverse sets of proteins. Most of them are glycoproteins, expressed on the cell surface of macrophages, endothelial cells, and smooth muscle cells. SR activity was first described when investigating the mechanism how macrophages are converted into lipid-containing foam cells found in atherosclerotic plaques (Goldstein *et al.*, 1979). A protein is defined as a SR if being able to bind modified low-density lipoproteins, and to act as “molecular

fly paper", i. e. binding to a broad range of ligands with high affinity. Most of the known SR ligands are polyanionic, but not all polyanionic molecules are ligands, indicating that there are structural and charge requirements for ligand binding. Several studies have indicated that SRs may play an important role in various physiological and pathological events, such as host defense processes. Based on the primary structure of the protein, the SR family is divided into different subgroups (Reviewed by Terpstra *et al.*, 2000). Class A is composed of SR-AI, AII, AIII and MARCO, and of two recently identified SR-A related proteins, SR-CL I and SR-CL II (SR with C-type lectin I and II) (Nakamura *et al.*, 2001). Another group has identified the same molecule, and termed it CL-P1 (Ohtani *et al.*, 2001). Class B consists of CD36, SR-B1/CLA-1, and the *drosophila* proteins croquemort and epithelial membrane protein (*emp*). The *drosophila* SR-C composes class C, whereas macrosialin/CD68 are the class D-receptors. Endothelial receptors LOX-1 and SREC are the class E-, and class F, respectively.

Scavenger receptors involved in innate immunity

Some of the SRs have been implicated in host defense as pattern-recognition receptors. In addition to SR-AI, SR-AII and MARCO, these include SR-CL I, SR-CL II/CL-P1 as well as dSR-C1, and LOX-1 (see figure 3). The structures of SR-CL I and II/CL-P1 resemble that of SR-A, with the exception that SR-CL I has a lectin-like domain instead of the cysteine-rich domain of SR-AI. SR-CL/CL-P1 is an endothelial receptor expressed on vascular endothelial cells and not in macrophages. SR-CL I and II/CL-P1 transfected CHO cells gain the ability to bind *E. coli* and *Staphylococcus aureus* (Nakamura *et al.*, 2001; Ohtani *et al.*, 2001). dSR-CI, expressed on drosophila macrophages, is able to recognize both gram-negative and gram-positive bacteria, but not yeast (Ramet *et al.*, 2001). LOX-1 is expressed in endothelial cells and macrophages, as well as in the smooth muscle cells of the atherosclerotic lesions (Kataoka *et al.*, 2000). Its expression can be induced by proinflammatory stimuli, such as TNF-α (Moriwaki *et al.*, 1998) and LPS (Nagase *et al.*, 1998). LOX-1 binds both gram-positive and gram negative bacteria (Shimaoka *et al.*, 2001).

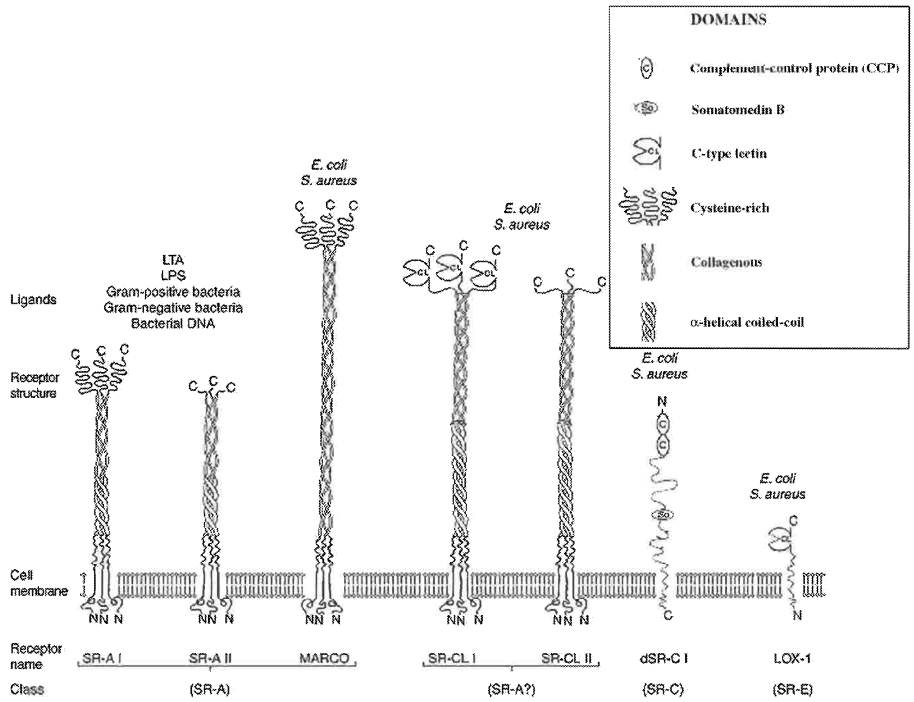

Figure 3. Schematic structures of scavenger receptors that have been implicated in microbial recognition. Adapted from Peiser *et al*. 2002.

Scavenger receptor AI and AII

The molecular identification of SRs began with the cloning of bovine class A scavenger receptor (SR-A) (Kodama *et al*., 1988). The gene was shown to generate two different forms through alternative splicing, SR-AI and II. A third naturally occurring splice variant of the human SR-A gene encoding a new isoform of the SR-A receptor, SR-AIII, has been described (Gough *et al*., 1998). SR-AIII has an altered C-terminal domain and is trapped in endoplasmatic reticulum. It acts as a dominant negative form of SR-A, suggesting involvement in the regulation of the SR-A activity. The SR-AI and II have been implicated in various macrophage-associated processes, such as host defense. They may also play a role in the formation of atherosclerotic plaques, as suggested in several reports.

Structure and proposed functions for the different domains

SR-AI and II are trimeric membrane proteins, which span the plasma membrane once and are orientated with the N-terminus in the cytoplasm. These two isoforms have five domains in common, i. e. cytoplasmic, transmembrane, spacer, α-helical coiled-coil and collagenous domains. In addition, SR-AI contains a scavenger receptor cysteine-rich (SRCR) domain (Kodama *et al.*, 1990). Electron microscopy after rotary shadowing and negative staining shows that the α-helical coiled-coil and collagenous domains are joined by a flexible hinge. The angle between these domains varies from 0 to 180 degrees (Resnick *et al.*, 1996). The collagenous region is required for ligand recognition, at least for the binding of modified lipoproteins, such as oxLDL or acLDL. It has been suggested that a cluster of four lysines in the most C-terminal part of the domain is essential for ligand binding (Doi *et al.*, 1993). However, one more recent report (Andersson and Freeman, 1998) raised some doubts about this concept, since several basic residues within the collagenous domain were found to be of importance for ligand binding. The α-helical coiled-coil domain is suggested to support both trimerization and acid-dependent ligand dissociation of SR-A (Doi *et al.*, 1993; Doi *et al.*, 1994). The interaction with intracellular proteins is mediated by the cytoplasmic domain of SR-A (Morimoto *et al.*, 1999).

Ligands

SR-AI and II bind a broad spectrum of ligands, of which all are polyanionic molecules (see table 2). These include acLDL, oxLDL, and maletylated BSA, but not their unmodified counterparts (reviewed in Krieger and Herz, 1994). Other known ligands for SR-AI and II are four-stranded polyribonucleotides, including polyinosinic acid (poly(I)) and polyguanosinic acid (poly(G)) (Pearson *et al.*, 1993). In addition, natural and modified polysaccharides, such as dextran sulphate and ficoll, are ligands of SR-A, as well as asbestos (Resnick *et al.*, 1993), LPS (Hampton *et al.*, 1991), and LTA (Dunne *et al.*, 1994).

Table II: SR-A ligands.

Ligand	Non-ligand
Oxidized Low Density Lipoprotein (oxLDL)	Native LDL
Acetylated LDL (acLDL)	
Oxidized High Density Lipoprotein (HDL)	Native HDL
Maletylated Bovine Serum Albumin (BSA)	BSA
Malondialdehyde BSA	
Fucoidan	Heparin
Dextran Sulfate	Chondroitan Sulfate
Polyguanylic acid (poly(G))	Polycytidylic acid (poly(C))
Polyinosinic acid (poly(I))	Polyadenylic acid (poly(A))
Crocidolite asbestos	
Silica	
LPS	
LTA	
Gram-negative bacteria	
Gram-positive bacteria	
Apoptotic cells	

Receptor recognition or nonrecognition has been shown for these molecules, either through direct binding studies or the inhibition of modified endocytosis. Adapted from Platt and Gordon 2001.

Expression and regulation of SR-A

SR-AI and II are constitutively expressed in tissue macrophages, e. g. Kupffer cells in liver, peritoneal macrophages and alveolar macrophages in lung (Emi *et al.*, 1993; Naito *et al.*, 1991). Perivascular macrophages surrounding arterioles in the brain, so called MATO cells, express SR-AI and II, whereas they are not expressed by microglia cells (Mato *et al.*, 1996). SR-AI and II are also found on sinusoidal endothelial cells in liver and adrenal gland (Naito *et al.*, 1991), and in the high endothelial cells of post capillary venules in lymph nodes (Geng and Hansson, 1995).

SR-A expression is markedly upregulated during maturation of monocytes to macrophages, and there is evidence for factors such as M-CSF being involved (de Villiers *et al.*, 1994). Furthermore, the expression and activity of SR-A were repoted to be down-regulated by LPS in the human monocyte-macrophage cell line THP-1 cells through TNF-α secretion (van Lenten and Fogelman, 1992). In contrast, SR-A expression is upregulated by LPS in both peritoneal macrophages as well as in such mouse cell lines as J774A.1 and RAW264.7 (Fitzgerald *et al.*, 2000).

Proposed functions of SR-AI and II

Stainings of atherosclerotic lesions demonstrated a highly positive staining for SR-A, indicating a role for SR-A in arteriosclerosis (Matsumoto *et al.*, 1990). Studies in SR-A knockout mice have further provided evidence for the involvement of SR-A in this vascular disease. Isolated peritoneal macrophages from SR-A-deficient mice have a significantly reduced capacity in uptake of modified LDL. However, the *in vivo* clearance by the liver is not affected. In line with the concept that SR-A plays a role in the formation of atherosclerotic plaques, apoE/SR-A-null mice have increased serum cholesterol levels and show significantly reduced lesion formation compared with apoE-null controls (Suzuki *et al.*, 1997). However, results from other models of arteriosclerosis have raised doubt about the generality of these findings. On an LDL receptor (LDLR)-deficient background, the absence of SR-A resulted in reduced plasma cholesterol levels, and only in a moderate lesion reduction (Sakaguchi *et al.*, 1998). Furthermore, the crossing of apoE3Leiden transgenic mice with SR-A knockout mice actually resulted in the development of more severe atherosclerotic lesions (de Winther *et al.*, 1999a). de Winther and colleagues (de Winther *et al.*, 1999b) also generated a transgenic mouse overexpressing SR-AI, and when crossed on to a LDLR-deficient background, SR-A overexpression resulted in a decreased atherosclerotic pathology.

Another set of studies suggest a function for SR-A in macrophage adhesion. These studies are based on the observation that the antimurine SR-AI and II monoclonal antibody, 2F8, inhibits cation-independent, serum-dependent adhesion of murine macrophages to plastic (Fraser *et al.*, 1993). Furthermore, when investigating the adhesion properties of BCG (bacillus Calmette-Guerin)-elicited peritoneal macrophages from SR-A knockout mice, Haworth and colleagues (Haworth *et al.*, 1997) found that the presence of EDTA completely inhibited adhesion to serum-coated plastic plates, wheras the adhesion of the wild-type cells remained. These results indicate that Ca^{2+} and Mg^{2+} are not required for the interactions of SR-A with its ligand. Moreover, SR-A -/- peritoneal macrophages display reduced adhesion and spreading over the first 24 hours after isolation (Suzuki *et al.*, 1997). SR-A is also able to mediate adhesion of macrophages to tissue ligands of frozen sections, since 2F8 antibody blocked adhesion of murine RAW264 macrophages to tissue sections such as spleen, lymph nodes, and lung

(Hughes *et al.*, 1995). Still, it has not been possible to demonstrate a role for SR-A in the macrophage adhesion/migration in mice. SR-A does not seem to be involved in the recruitment of macrophages to BCG-induced granulomata, or to the peritoneal cavity after *S. aureus* administration, since there was no significant difference in the amount of activated macrophages in the SR-A null mice and controls (Haworth *et al.*, 1997; Thomas *et al.*, 2000).

There are also findings indicating a potential role for SR-A in cell-cell interactions. Namely, it was found in one study that activated B-cells, but not resting B-cells, adhere to stably transfected CHO cells (Yokota *et al.*, 1998). Interestingly, this adhesion was not inhibited by the known SR ligands, fucoidan or acLDL. As speculated in the paper, this interaction can play a role in the recruitment of activated B-cells to lymph nodes and the sites of infection.

Apoptotic cell recognition may be a universal property of SRs. At least this property has been demonstrated for all SRs examined so far, including those of invertebrates (Platt and Gordon, 1998). *In vitro* experiments show that SR-A can recognize at least two dying cell populations, apoptotic thymocytes and activated platelets. In both cases, 2F8 inhibits this binding significantly (Platt *et al.*, 1998). Furthermore, SR-A-deficient thymic macrophages showed 50% reduction in the phagocytosis of apoptotic thymocytes. However, extensive studies on thymocyte clearance did not demonstrate any significant defect in SR-A knockout mice (Platt *et al.*, 2000).

SR-A is expressed on microglia during different forms of injury in CNS (Bell *et al.*, 1994). This could be an indication that SR-A is involved in neuronal disorders. There is also evidence for SR-A expression on microglia surrounding amyloid plaques in Alzheimer disease (Christie *et al.*, 1996). Furthermore, *in vitro* studies show that the microglial endocytosis of the Aβ peptide, that appears to play a key role in the pathogenesis of Alzheimer disease, can be inhibited with the ligands of SR-A (Huang *et al.*, 1999). Moreover, interactions between SR-A and Aβ aggregates seem to exhibit the release of neurotoxins (El Khoury *et al.*, 1996). Recently however, the importance of SR-A involvement in Alzheimer disease has been questioned, since studies with the transgenic mice expressing human amyloid precursors did not show any reduction in the

formation of amyloid plaques when eliminating SR-A (Huang *et al.*, 1999). In addition, Antic et al (Antic *et al.*, 2000) reported that Aβ does not block the binding to SR-A ligands in peripheral blood monocytes (PBMs), either did the ligands stimulate neurotoxin release.

Two lines of evidence indicate a role for SR-A in the innate immune defense; SR-A is expressed primarily on macrophages (Hughes *et al.*, 1995), and it binds both gram-negative and –positive bacteria, as well as their surface components LPS and LTA, respectively (Krieger and Herz, 1994; Pearson, 1996). SR-A is also upregulated in the macrophages of BCG-induced granulomata, and in mouse brain in response to LPS injection (Bell *et al.*, 1994; Haworth *et al.*, 1997). The crucial data supporting an *in vivo* role for SR-A in host defense have come from the studies of SR-A deficient mice. SR-A -/- mice were reported to be more susceptible to infection with *Listeria monocytogenes*, *S. aureus*, and the DNA virus Herpes simplex virus type-1 compared to wild-type littermates (Suzuki *et al.*, 1997; Thomas *et al.*, 2000).

SR-A knockout mice have also been used to elucidate the role of SR-A in endotoxin shock. One study provided evidence that BCG-infected mice lacking SR-A show approximately sevenfold higher mortality compared to wild-type mice after LPS injection. SR-A null mice were also producing more TNF-α and IL-6 in response to LPS-challenge than the control animals (Haworth *et al.*, 1997). These results indicate that SR-A plays a protective role in the uptake and cellular response to LPS. Contradictory results were presented by Kobayashi *et al.* (Kobayashi *et al.*, 2000), who found the SR-A-deficient mice to be more resistant to LPS compared to the wild-type mice, when challenged with LPS intraperitoneally. In addition, IL-1 production was found to be impaired in the SR-A null animals.

Recent studies have also provided evidence for SR-A involvement in initiating the adaptive immune response. Nicoletti et al (Nicoletti *et al.*, 1999) have reported that dendritic cells express SR-AI and II, and that they can mediate the uptake of modified LDL. It turned out that, unlike wild-type littermates, the SR-A -/- mice cannot mount a measurable immune response to the SR ligand maletylated albumin.

MARCO

The identification of MARCO was a "cloning accident" which occurred when screening a mouse macrophage library in λgt11 for type XIII collagen in 1990. The receptor turned out to be a transmembrane protein with a long collagenous part. The full-length mouse MARCO was cloned, and was found to be highly homologues with the recently cloned protein SR-A (Elomaa *et al.*, 1995; Kodama *et al.*, 1988).

Structure

Mouse MARCO is a triple-helical transmembrane protein consisting of 518 amino acid residues. It is composed of five different domains. The intracellular domain is a 49-residue hydrophilic domain containing one cysteine (the results of paper I show evidence for another form which has a shorter cytoplasmic domain). MARCO has one hydrophobic stretch of 25 amino acids, the transmembrane domain, which is followed by a hydrophilic spacer domain containing 75 residues including two cysteine residues and two putative N-glycosylation sites. The collagenous domain is a 270-residues long. The collagenous Gly-Xaa-Yaa-repeat is interrupted at one location by the sequence Ala-Glu-Lys, suggesting a hinge region in the triple helix of the MARCO molecule. The C-terminal SRCR domain, also termed domain V, has 99 residues, six of which are cysteines (Elomaa *et al.*, 1995).

The human MARCO was found to be highly similar to the mouse molecule (Elomaa *et al.*, 1998). It consists of 520 amino acid residues. The identity of the amino acid sequence between the full-length human and mouse polypeptide chains is 68%. The sequence identity of the chains between the two species is highest in the SRCR and, as expected, in the collagenous domain, which has a glycine residue at every third position. Between the intracellular domains, the sequence identity is surprisingly low, only 51%. Interestingly, the human MARCO subunit chain differs from the mouse chain in that it does not have any cysteine residues outside the SRCR domain, which is also the case for the recently described hamster MARCO (Palecanda *et al.*, 1999). The two cysteine residues in the spacer domain of mouse MARCO participate in the formation of interchain disulfide bonds in the trimeric MARCO molecule (Elomaa *et al.*, 1995). Thus,

the three subunit chains of the human and hamster MARCO are bound to each other only through the collagenous triple helix, as the cysteine residues in the SRCR domain all form intrachain bonds. The potential glycosylation sites in the spacer domain, the interruption in the collagenous domain, and the six cysteines in the SRCR domain are all conserved between the three species, suggesting that these features are important for the structure/function of MARCO.

Comparison between MARCO and SR-AI structures

The overall structure of MARCO is very similar to that of SR-AI. Both are trimeric transmembrane type II receptors, with an N-terminal intracellular portion and the C-terminal located extracellularly, as well as with a single membrane-spanning domain. The type II membrane orientation occurs only in 5% of plasma membrane proteins (Singer, 1990). The triple-helical structure differs between the two receptors. MARCO consists of a long collagenous domain, interrupted at one site, whereas SR-AI and II are composed of a noncollagenous α-helical coil-coiled domain followed by a short uninterrupted collagenous domain. The SRCR domain is similar in SR-AI and MARCO. This domain also exists in several other proteins. The only known function is the one in CD6, which is a T- and B-cell surface protein. The SRCR domain of CD6 interacts with ALCAM (activated leukocyte cell adhesion molecule) (Whitney *et al.*, 1995), which is an immunoglobulin superfamily protein on thymic epithelial cells and activated leucocytes (Bowen *et al.*, 1995). The intracellular portion of MARCO contains no known phosphorylation sites (Elomaa *et al.*, 1995). In comparison, the cytoplasmic domain of SR-A consists of three potential phosphorylation sites, two serines and one threonine (Ashkenas *et al.*, 1993).

Tissue distribution

MARCO expression is restricted to macrophages. Its expression has been studied most extensively in mouse with the help of a panel of monoclonal antibodies. In unstimulated mice, MARCO is expressed by macrophages in the marginal zone of the spleen, on macrophages of the medullary cords in lymph nodes, and on peritoneal macrophages (Elomaa *et al.*, 1995). Ito and collegues (Ito *et al.*, 1999) claim that the only MARCO-

positive cells are endothelial cells of the marginal sinuses and that MARCO-expressing macrophages appeared in the lymphatic sinuses after LPS injection. During mouse embryonic development, MARCO is expressed in the spleen from day E17 onwards as detected by immunohistochemistry. However, using northern blot analysis, MARCO mRNA expression could already be detected at day E15 (Elomaa unpublished observation). This expression pattern appear both in BALB/c mice and C57BL/6 (Elomaa *et al.*, 1995; Ito *et al.*, 1999).

A more detailed study of the macrophages in the marginal zone of the spleen in op/op mice and BALB/c controls showed that marginal zone macrophages include MARCO-positive, ER-TR9-positive and doubly-positive subpopulations in controls as well as in op-/op+ littermate control mice (Ito *et al.*, 1999). op/op mice, that are defective in production of M-CSF (Yoshida *et al.*, 1990), do not have some subpopulations of marginal zone macrophages (Takahashi *et al.*, 1994). It turned out that these mice possess only MARCO-positive marginal zone macrophages (Ito *et al.*, 1999), suggesting that this subpopulation is M-CSF-independent as are the dendritic cells, which are present in normal numbers in op/op mice (Takahashi *et al.*, 1993).

In vitro, the mouse macrophage cell line IC-21 express detectable levels of MARCO (Elomaa *et al.*, 1995).

Expression of MARCO during inflammation

In vivo experiments demonstrate that MARCO expression is induced in several subpopulations of tissue macrophages in response to various inflammatory conditions, suggesting an important role in host defense. Expression can be observed on macrophages in liver and red pulp of the spleen 45 minutes after administration of heat-killed *E. coli*. Living *E. coli* or *S. aureus* can also induce MARCO expression (van der Laan *et al.*, 1999). In addition, acute endotoxic shock induced by *Klebsiella pneumonia* instillation in the lung results in a dramatic upregulation of MARCO on subpopulations of macrophages, such as Kuppfer cells and alveolar macrophages. These cells do not normally express this receptor. This expression of MARCO is reported to be transient and declines over a period of weeks after recovery (van der Laan *et al.*, 1997). Furthermore,

LPS can induce MARCO expression. With a dose of 25 μg of LPS, MARCO-positive macrophages are observed in liver, lung, and red pulp of the spleen after 6 hours (van der Laan *et al.*, 1999). After administration of BCG, *L. monocytogenes*, and zymosan both Kuppfer cells and spleen red pulp macrophages expressed MARCO (Ito *et al.*, 1999).

In situ hybridization, carried out on several human tissues obtained from two newborn infants who died from sepsis, revealed expression in macrophages in several tissues. In thymus, the expression was observed in scattered cells of the stromal compartment. In intestine, MARCO was expressed in cells located beneath mucosal membrane, and in kidney, positive cells is observed in the interstitium. Intense staining was seen in liver, mainly in Kuppfer cells. In spleen, the signals were detected in the white pulp region. No transcripts were detected in lung. In all tissues examined, the expression was exclusively observed in tissue macrophages (Elomaa *et al.*, 1998).

In vitro studies showed that addition of LPS to the mouse macrophage cell line J774.2 readily upregulated MARCO expression in the presence of serum. However, incubations with proinflammatory cytokines such as IFN-γ, IL-6, TNF-α, or IL-1 did not lead to upregulation (van der Laan *et al.*, 1997), suggesting that LPS binding itself can activate the cells.

Binding properties of MARCO

MARCO-transfected cells readily bind FITC-labeled heat-killed *E. coli* and *S. aureus*, but not yeast cells (zymosan). The neutral polysaccharide Ficoll, which is avidly taken up by marginal zone macrophages, does not show any binding to MARCO-positive cells (Elomaa *et al.*, 1995). AcLDL is a ligand for mouse MARCO (Elomaa *et al.*, 1995), but not for human MARCO (Elshourbagy *et al.*, 2000), suggesting diverse function of the receptor in the two species. The physiological significance of the affinity of mouse MARCO for acLDL remains unclear. Whether oxLDL is a ligand of MARCO has not been tested. Immunohistochemical stainings on atherosclerotic lesions show that MARCO is not expressed in lipid-laden foam cells in contrast to SR-AI and II (Elomaa, 1996; Matsumoto *et al.*, 1990). Consequently, MARCO is likely not having a major role in lipid metabolism. Recently, MARCO was identified as a major receptor on alveolar macrophages (AMs) for binding unopsonized environmental particles, such as TiO_2 and

Fe_2O_3 (Palecanda *et al*., 1999). Uptake of unopsonized TiO_2 and scavenger receptor ligands, such as poly(I) and acLDL, cause no significant activation of AM respiratory burst or TNF production (Kobzik, 1995). This could be especially useful in clearance of inert dusts and particles, because they could be removed without risk of the potentially damaging effects of an inflammatory response in the lung.

OUTLINES OF THE PRESENT STUDY

At the outset of this study the full-length mouse and human cDNAs had been cloned in our laboratory. These clones had been used to express MARCO in transfected cells and the MARCO-positive cells had been found to gain the ability to bind heat-killed bacteria and acLDL (Elomaa *et al*., 1995). Inspired from those data plus the interesting expression pattern, our group has since then pursued studies to elucidate the role of MARCO in anti-microbial host-defense mechanisms. Recently, MARCO was also identified as the major receptor on alveolar macrophages for binding unopsonized environmental particles (Palecanda *et al*., 1999).

One of the aims of the present study was to further characterize MARCO, its binding properties and possible other properties important for the host-defense functions. Another aim was to elucidate the structure of the MARCO gene. This information will be useful if MARCO can be connected to some genetic disease and for generation of knockout mice. Knowledge about the gene structure might also be of use when trying to understand the gene regulation of MARCO.

The specific aims were:

* To determine the structure of the human and mouse genes.
* To explore the unusual morphological changes induced in cells transfected with MARCO.
* To determine the bacteria-binding region of MARCO.
* To identify ligands of MARCO

METHODS

The methods used in this thesis are described in detail in the "Materials and Methods" section of each paper. Here there is a brief overview of some of the methods used .

Isolation of genomic clones **(Paper I)**

We made ^{32}P-labeled probes from human MARCO cDNA (Elomaa *et al.*, 1998), and screened two λ-phage libraries (Clontech HL1067j and HL1111j) (Sambrook J., 1989) for the isolation of the human MARCO gene. The mouse gene was isolated from a BAC mouse ES cell library (Genome systems, Inc.) using a probe from the mouse cDNA (Elomaa *et al.*, 1995) in hybridization screening. The clones were characterized using restriction mapping, Southern hybridization, and PCR.

Identification of the transcription initiation site **(Paper I)**

Primer extension and S1 nuclease mapping were used to define the transcription initiation sites for mouse MARCO. Total RNAs from the mouse macrophage IC-21 (ATCC TIB 186) cell line or LPS-activated J774A.1(ATCC TIB 67) mouse macrophages were used as a template. The IC-21 cells are known to express MARCO (Elomaa *et al.*, 1995). J774A.1 cells express MARCO if stimulated with LPS. Raw 264.7 (ATCC TIB 71) mouse macrophages, which do not express MARCO, were used as controls.

In primer extension, a ^{32}P-labeled MARCO-specific primer was used in a reverse transcription reaction. RNase treatment depleted the RNA. For S1 nuclease mapping, a genomic probe radioactively labeled with ^{32}P was used. The 188 nucleotide probe extended 122 base pairs upstream of the major transcription site. It was hybridized to the isolated mRNA, and then the unprotected single-stranded RNA and DNA were digested with S1 nuclease. The products were analysed on a 6% polyacrylamide/8M urea gel and exposed to an X-ray film, since the agarose gel does not separate small fragments efficiently. As a complementary approach to the first two assays we used 5´RACE to identify the sequence of the 5´end region of the MARCO mRNA. We made a full-length cDNA library using total RNA from spleen or cultured macrophages as a template. The first-strand synthesis was performed using an oligo(dT) primer and reverse transcriptase

(Gibco BRL). A SMART oligonucleotide that captures the 5´CAP structure was also included in the reaction. PCR amplification was perfomed using SMART 5´primer and the 3´primer according to the kit´s protocol. Amplification of the 5´end of MARCO was made using the SMART 5´primer and a MARCO-specific antisense primer using the amplified cDNA as a template. The PCR products were purified and sequenced.

Fluorescence in situ hybridization (FISH) **(Paper I)**

Chromosomal assignment of the human and murine MARCO genes were carried out in collaboration with Y. Matsuda, R. Eddy, and T. B. Shows using FISH on metaphase spreads with genomic fragments as probes (Feinberg and Vogelstein, 1983; Matsuda and Chapman, 1995; Matsuda *et al.*, 1992; Seldin, 1994; Trask, 1991). The probes were labeled either with digoxygenin-11-dUTP (Boehringer Mannheim) using random priming or with biotin-16-dUTP (Boehringer Mannheim) and nick translation.

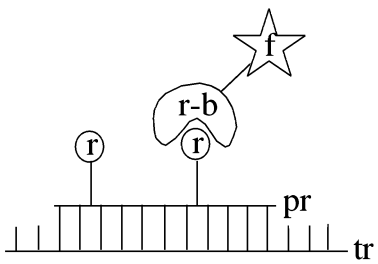

Figure 4. Fluorescence in situ hybridisation (FISH). Abbreviations: f, fluorochrome; pr, probe labeled with reporter molecule; r, reporter molecule (e. g. biotin, digoxigenin); r-b, reporter-binding molecule (e. g. avidin, streptavidin, or digoxegenin antibody); tr, transcript. (Adapted from Current Protocols in Molecular Biology)

Expression Constructs **(Papers II-IV)**

All MARCO expression constructs used in transient transfections were cloned into the expression vector pcDNA3 (Invitrogen). pcDNA3 vector contains the strong cytomegalovirus promotor and enhancer region, which gives high expression of the construct of interest in mammalian cells. The truncated and point-mutated constructs were generated by site-directed insertion of a stop codon using PCR or Quick change site-directed mutagenesis kit (Stratagene). The constructs encoding proteins with large internal deletions, recombinant soluble MARCO (sMARCO), or recombinant domain V

(recV) were made using restriction enzymes and PCR according to established molecular biological methods. sMARCO and recV were cloned into the expression vectors pCEP-Pu and pCEP-Pu/AC7, respectively (kindly provided from T. Sasaki and R.Timpl, Max Planck Institute for Biochemistry, Martinsried, Germany). These vectors are maintained extra-chromosomally and express high levels of recombinant proteins. They contain the EBV origin of replication. When transfected into the 293/EBNA cells, that constitutively express its nuclear antigen, the vectors replicate episomally in multiple copies. All produced construct were sequenced by the ABI 310 DNA sequencer (Perkin Elmer). The various constructs are summarized in schematic forms in papers II-IV.

Transient transfections **(Papers II-IV)**

CHO (Chinese hamster epithelial cells), HeLa (human epithelial cells), NIH3T3 (mouse fibroblast cells), and 293 (human epithelial cells) were transfected using the calcium-phosphate method. In this method, a precipitate containing calcium phosphate and DNA is first formed in an HEPES-buffered saline solution. The precipitate adheres to the cells and is incorporated by an unknown mechanism. There are at least two factors that influence the efficiency of this method, the amount of DNA and the time the precipitate is left on the cells. In some cell types the transfection efficiency is dramatically increased by glycerol or DMSO shock. We used 20 μg of DNA per a 100-mm dish. In co-transfections, 10 μg + 10 μg of the expression plasmids were used. Precipitates were incubated on cells overnight, and cells were seeded on glass coverslips 24 h after transfection. In some experiments of paper II, transfected cells were taken into suspension 24 h after transfection, and were incubated in suspension for 12-16 h before plating on uncoated coverslips or on coverslips coated with FCS, fibronectin, laminin-1, or vitronectin.

Stable transfection **(Paper IV)**

To produce cell clones that constitutively express the gene of interest, the gene is generally transfected into a cell line together with a gene expressing a selectable marker. We used L929 cells, (mouse fibroblast cells (ATCC)) that were transfected with the plasmids pcDNA3-mouse MARCO (Pikkarainen *et al.*, 1999) and PGK-Puro as a selection marker. The stable clones were selected in the presence of G418 and

puromycin. Stable clones were also generated for the production of sMARCO and recV (see "*Production and purification of sMARCO and recV*").

Analysis of protein expression **(Papers II and III)**

In paper II, cell surface proteins were labeled by incubating cells with sulfo-NHS-biotin in the ice-water bath. Only cell surface proteins are labeled since sulfo-NHS-biotin does not penetrate into the cells due to the negative charge of the sulfo-group. Biotinylated proteins were precipitated from the cell lysates with streptavidin-agarose. The precipitated proteins were eluted by boiling and the MARCO proteins were detected using Western blotting with the anti-MARCO antibodies.

In some assays, we estimated cell surface levels of MARCO by antibody staining (Paper III). Living cells were incubated on ice with the antibody before fixation and incubation with a FITC-labeled secondary antibody.

Immunofluorescence **(paper II-IV)**

For immunofluorescence analysis, cells grown on glass coverslips were fixed in a paraformaldehyde solution, and then permeabilized in a PBS solution containing Triton X-100. Free aldehyde groups were quenched by incubating cells in PBS containing 50 mM NH_4Cl. Thereafter, cells were incubated in a blocking solution, such as 2% bovine serum albumin or 10% goat serum, if the secondary antibody is made in goat. When staining for mouse MARCO, the cells were incubated either with rat anti-mouse monoclonal antibodies 1.18 or 7.21 (also known as ED31) (kindly provided by L. van der Laan and G. Kraal, Vrije university, Amsterdam, The Netherlands), recognizing intracellular and the SRCR domains, respectively. Polyclonal antibodies raised against domain I and domain V of the mouse MARCO protein (Elomaa *et al.*, 1995) were also used in paper III. The cells expressing human MARCO constructs were identified using a new polyclonal antibody raised against the intracellular domain of human MARCO. We also used a mouse monoclonal antibody against paxillin (Transduction laboratories) to recognize focal adhesion structures. As secondary antibodies, we used FITC-labeled rabbit anti-rat IgG (DAKO), biotinylated rabbit anti-mouse IgG, goat anti-rat Alexa Fluor 546-conjugated IgG, FITC-labeled swine anti-rabbit IgG, and TRITC-conjugated swine anti-rabbit IgG (DAKO). Coverslips were mounted into PBS containing glycerol and p-

phenylenediamine (for anti-fading) or polyvinylalcohol (Movioil; Hoechst) without anti-fading factors. For filamentous actin staining, rhodamine-conjugated phalloidin (Molecular Probes) was added to the secondary antibody solution.

Generation of antibodies against the domain I of human MARCO **(Paper III)**

The intracellular domain (domain I) was expressed in *E. coli* as a glutathione S-transferase (GST) fusion protein using the pGEX-1lT vector (Pharmacia). A DNA fragment encoding domain I was generated by PCR. Fusion proteins are generally created by a translational fusion of the coding sequence for the protein of interest to a gene of a highly expressed protein partner (or "carrier" protein). C-terminal protein fusions to GST are usually soluble and well-expressed (Smith and Johnson, 1988). GST binds specifically to glutathione, and the intracellular domain was cleaved from the fusion protein bound to the glutathione-agarose beads by incubation with thrombin, according to Pharmacia's instructions. Following cleavage, the MARCO polypeptide was eluted from the column, and used to raise antisera in rabbits. IgG was purified from the antisera by protein A-Sepharose (Pharmacia). The antibodies were found to stain transfected cells expressing human MARCO. They also detected human MARCO in Western blots.

Bacteria-binding assays **(Paper III and IV)**

The transfected cells were washed, then incubated in DMEM containing the heat-killed FITC-labeled *E. coli* (Molecular Probes) (Paper III), or FITC-labeled living bacteria (Paper IV) for 45-60 min in a humidified atmosphere with 5 % CO_2 at 37°C. After this, the cells were washed, fixed with 4 % paraformaldehyde, and stained with anti-MARCO antibodies. When assaying the bacteria-binding activity of sMARCO and recV, coverslips were first coated with recombinant proteins. Then they were incubated in a blocking solution containing BSA, followed by the incubation with fluorescently labeled bacteria as described above. In some experiments, the transfected cells or sMARCO-coated surfaces were incubated with the known scavenger receptor ligand poly(I) or known non-ligands poly(C) or heparin before or during the bacterial incubation. The capacity of the variants of the MARCO receptor for binding bacteria was estimated semiquantitatively by fluorescent microscopy.

Production and purification of sMARCO and recV **(Paper IV)**

293/EBNA cells were transfected using the calcium-phosphate method (see "*transient transfection"* above) with the sMARCO or recV constructs. The stable clones were selected with puromycin. The production of recombinant proteins was tested by dot-blot analysis. sMARCO was purified from serum-free medium using the Ni-NTA beads and eluted with PBS containing imidiazole. RecV was applied in a cation exchange column and eluted with a linear NaCl gradient (90-249 mM). After this, the sample was passed over a phenyl-sepharose column and eluted with a linear $(NH_4)_2SO_4$ gradient (1.8-0 M).

Circular Dichroism Analysis **(paper IV)**

One of the most commonly used techniques to study secondary structures of proteins is the circular dichroism (CD). Purified sMARCO was dialyzed against a buffer, at 30.6 µg/ml, and the CD spectra was recorded on a AVIV Model 62DS CD^2 spectrophotometer using a thermostatted cuvette with a path length of 0.1 cm. Melting curves were recorded by measuring the molar ellipticity at the wavelength 220 nm between 15 °C and 70 °C, increasing the temperature by 30 °C/h. The concentration of sMARCO was determined by amino acid analysis.

Electron Microscopy **(Paper IV)**

Shadowing experiments use heavy metals to amplify the low intrinsic contrast of proteins. The metal is applied by evaporation to form a thin coating on predried specimens. Images of rotary-shadowed specimens tend to be easier to interpret because they contain fewer lacunae where no metal grains reach than unidirectionally shadowed images. sMARCO molecules were mixed with equal volumes of 80% glycerol and sprayed onto freshly cleaved mica pieces with a nebulizer designed for small volumes shortly after mixing. Glycerol has a conformation-preserving effect and is especially applied to filamentous proteins. They were dried in high vacuum and shadowed under rotation with platinum/carbon at a 9° angle. Low-angle rotary shadowing has proved particularly effective for the study of filamentous proteins. Specimens were observed in a LEO EM 910 electron microscope operated at 80 kV accelerating voltage. Length measurements and statistical evaluations were performed with a personal computer attached to a graphics tablet.

LPS-binding assays **(Paper IV)**

We used two different assays to study whether MARCO is capable of binding LPS. L929-cells expressing the full-length mouse MARCO were rinsed with serum-free DMEM, and incubated with FITC-labeled LPS (Molecular Probes) in serum-free DMEM for 1 hour at 37 °C. Following incubation, cells were washed five times with PBS and fixed in paraformaldehyde. Binding was evaluated by fluorescent microscopy. The other assay was carried out using sMARCO that was incubated with LPS (Sigma) in 30 min at room temperature with constant mixing. After incubation, Ni-NTA beads were added, and the incubation was continued for 1 h with constant mixing. After centrifugation, the beads were washed with PBS, boiled in the SDS-PAGE sample buffer under reducing conditions, and analyzed by SDS-PAGE. LPS and glycoproteins were detected by staining with the Pro-Q™ emerald 300 Lipopolysaccharide Gel Stain Kit (Molecular Probes).

RESULTS AND DISCUSSION

Cloning and chromosomal localization of the MARCO genes (Paper I)

We cloned the mouse MARCO gene from a genomic mouse BAC library and the human MARCO gene from four genomic λ phage clones. The mouse and human MARCO gene have a size of 30 and over 50 kilobases, respectively. In addition, the mouse gene was localized to chromosome 1 and the human gene to a syntenic region on chromosome 2 by fluorescent *in situ* hybridization (FISH). The results will facilitate studies on the potential involvement of MARCO in genetic diseases affecting the host defense. However, at present no such human diseases have been localized to the MARCO gene locus on chromosome 2. Furthermore, the mouse gene structure is a prerequisite for the generation of knockout mice that have been generated in our laboratory (unpublished data). Both genes have a similar overall structure with 17 exons. The exon-intron structure of the MARCO receptor gene corresponds in most cases to the domain structure (see figure 5). A similar exon-domain correlation is found in the gene of the close structurally related SR-A (Emi *et al.*, 1993). Sequences at the exon-intron junctions of all 17 exons are compatible with the slice consensus sequences, including the highly conserved dinucleotides AG-GT (Shapiro and Senapathy, 1987). Exons 4-15 code for the collagenous domain. They are all exact multiples of 9 bp, as is typical also for fibrillar collagen genes (Drickamer and McCreary, 1987; Vuorio and de Crombrugghe, 1990). The cysteine-rich domain V is encoded by the last two exons, which is also the case for SR-AI (Emi *et al.*, 1993).

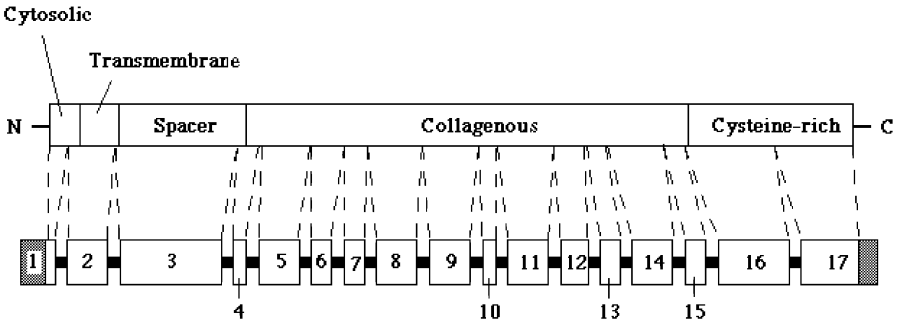

Figure 5. Structural relationship of exons with functional domains in MARCO. (Top) Structural domains, (Bottom) Coding regions of exons (boxes) are depicted by dashed lines.

The transcription initiation sites were identified by primer extension, 5´capturing RACE, and S1 nuclease mapping. Identical results were obtained when analyzing mRNA from two different cell lines, mouse macrophage cell line IC-21 expressing MARCO constitutively, or LPS-activated J774A.1 mouse macrophages. The major transcription initiation site is at position +1, 27 bp downstream of a TATA box. Other frequently used sites are at positions –63 and –66 downstream of an AT-rich region (see figure 6). Since the 5´ end of the MARCO gene is well conserved between mouse and human, it is likely that the same ATG codon is used as the main translation initiation codon in both species. The results indicate that the main form of cytoplasmic domain in both human and mouse MARCO consists of 20 residues, instead of the previously reported 49 residues in mouse receptor (Elomaa *et al.*, 1998). It is noteworthy that SR-AI and II have a cytoplasmic domain of 55 residues (Kodama *et al.*, 1990).

To initially characterize the regulatory elements of the MARCO gene, we searched for potential binding sites for transcription factors within the promotor region (see figure 7). The sequences of the 5´ends and the upstream regions of the human and mouse MARCO genes are quite conserved. Both have a TATA box and two conserved in-frame ATG codons for the initiation of translation in agreement with the Kozak consensus sequence (Kozak, 1989). A TATA box is usually observed in genes that are expressed in a cell-type specific pattern. Interestingly, the SR-A gene lacks a TATA box,

but it has an AT-rich region (Moulton *et al*., 1992). Expression of MARCO is induced in mice during systemic bacterial infections and by LPS (van der Laan *et al*., 1997). NFκB, ETS and C/EBP families are known to be LPS-responsive transcription factors (Sweet and Hume, 1996). We identified potential binding sites for three ETS family members in both genes, PU.1, PEA3 and Ets-1. PU.1 is a candidate for mediating macrophage-specific gene expression, because it is only expressed in macrophages and B-cells (Klemsz *et al*., 1990). Potential sites for NFkB and C/EBP exist only in the mouse gene. The functional significance of these potential regulatory elements in the MARCO genes remains to be determined. In the case of SR-A, a 291 bp fragment of the proximal promotor region is together with a 400 bp upstream enhancer element sufficient to target macrophage-specific expression in transgenic mice (Horvai *et al*., 1995). SR-A expression is dependent on cooperation between Ap-1 and ets-domain transcription factors (Wu *et al*., 1994). Moulton et al (Moulton *et al*., 1994) also demonstrated that PU.1 is important for macrophage-specific expression of human SR-A, whereas the mouse promotor does not seems to require PU.1 (Aftring and Freeman, 1995).

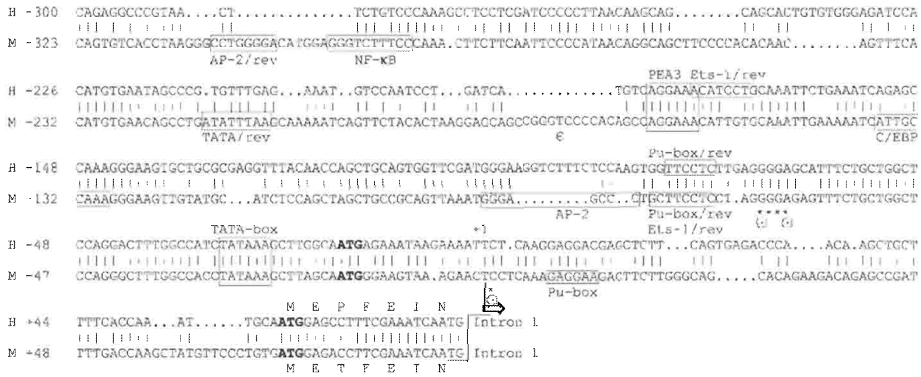

Figure 6. Nucleotide sequence of the 5´ end and the immediate upstream region of the human (H) and mouse (M) MARCO genes. Nucleotide position +1 is assigned to the major transcription initiation site in mouse (arrow). Transcription initiation site determined by the S1 nuclease assay and by 5´RACE are marked by an asterisk and white dots, respectively. TATA element and other putative regulatory elements are boxed and labeled. ATG codons that agree with the Kozak are in boldface type.

MARCO induces dramatic morphological changes in different cell lines (Paper II)

Ectopic expression of MARCO induces dramatic cell shape changes in such cell lines as CHO, HeLa, NIH3T3, and 293. Forty hours after transfection, MARCO-expressing cells often have processes with lengths several times the size of the cell body (see figure 7). COS7 cells responded somewhat differently, and these cells formed short pine needle-like protrusions. COS cells adhere very tightly to the underlying surface, and this might be the reason why MARCO does not induce dramatic cell shape changes in this cell line. In agreement with this possibility is the observation that 293 cells, which develope the longest processes of the cell lines tested, adhere poorly. The process formation is not due to overexpression of a membrane protein since the transient expression of another type II membrane protein did not induce cell shape changes. Furthermore, MARCO induce morphological changes when expressed under a weak promotor. We have also performed transient transfection experiments with SR-AI, and this protein was not observed to induce dendritic extensions (unpublished data). The MARCO-induced morphological

changes are accompanied by the rearrangement of the actin cytoskeleton. Non-transfected CHO cells have both focal adhesions and stress fibers, while these structures are substituted in MARCO-expressing cells by fine actin filaments, extending from the cell periphery into the processes. The extensions turned out to be ”protrusive filopodia” and not trailing tails, remained from migratory cells. The MARCO-specific cell shape changes can be observed already a few minutes after plating from a suspension culture. The cell spreading is fairly symmetrical and lamellipodia formation appear in all directions, but the long processes formed after the spreading are polarized. Extracellular matrix (ECM) components, such as laminin-1 and vitronectin do not prevent the MARCO-induced morphological changes, wheras fibronectin clearly inhibits the formation of extensions. In line with this finding, cell adhesion to fibronectin have been reported to promote actin stress fiber and focal adhesion formation (Burridge and Chrzanowska-Wodnicka, 1996).

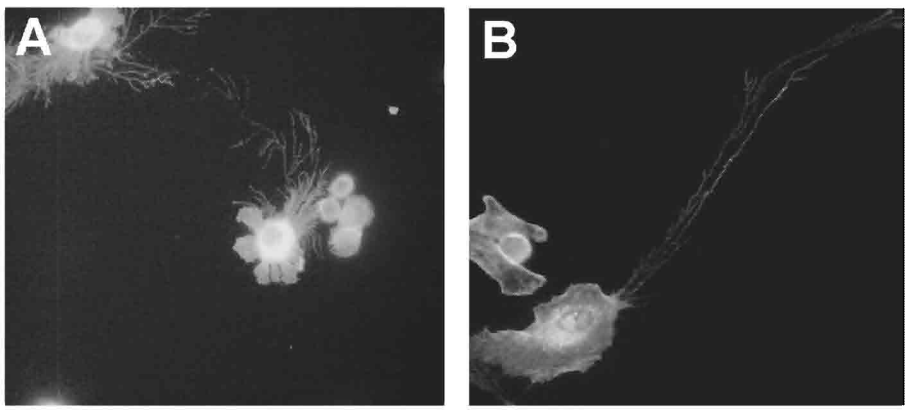

Figure 8. Expression of MARCO induces formation of long plasma membrane processes. CHO (A) and HeLa cells (B) were transiently transfected with an expression plasmid encoding the full-length MARCO and examined 40 h after transfection. Cells were double-stained for MARCO (FITC) and filamentous actin (rhodamine).

RESULTS AND DISCUSSION

We used truncated MARCO forms to determine that the first 15-20 residues of domain V are important for the morphoregulatory activity. This is based on two observations. First, a MARCO form that completely lacks domain V does not change cell shape even though it is expressed at high levels at the cell surface. Second, a form that has 16 residues of domain V is as active as the full-length MARCO in promoting morphological changes of cells. It is possible that MARCO binds some serum components coated to the glass, and interaction with the ligand(s) induces these dramatic cell shape changes. In agreement with this observation, MARCO-transfected CHO cells change their morphology when plated on anti-MARCO antibody-coated plate (unpublished data). Another possibility would be that the cell shape changes are dependent on the interaction of MARCO with another cell surface protein. Indeed, cells expressing the integrin $\alpha_{6A}\beta_1$ were found to induce filopodia formation and migration without binding its ECM ligand. Instead, these morphological changes seem to depend on the association of the integrin with another cell surface molecule, CD81 (Domanico *et al.*, 1997). It is noteworthy in this context that SR-A has been shown to function as an adhesion protein, whose ligand is present in fetal calf serum (Fraser *et al.*, 1993). In addition, thioglycollate-elicited peritoneal macrophages from SR-A knockout mice have been found to adhere to glass slower than wild-type macrophages (Suzuki *et al.*, 1997). A dominant-negative form of the small GTPase Rac-1 inhibited the MARCO-induced cell shape changes in CHO cells, but not in HeLa cells. Rac-1 is known to promote cell spreading and lamellipodia formation, but it does not induce formation of long plasma membrane processes (Hall, 1998), indicating that Rac-1-pathway is not the only pathway activated in the MARCO expressing CHO cells.

In conclusion, MARCO-expression induces cell shape changes that are specific to this type II membrane protein. This activity could be important for enlarging the cell surface area, to facilitate the capture of foreign particles, such as invading pathogens. The morphological changes observed in the MARCO-expressing cells in culture could also reflect the importance for MARCO in phagocytosis. The long plasma membrane extensions induced by MARCO could ”embrace” large particles very efficiently. In this context, it is of interest to note that MARCO is expressed on highly phagocytic macrophages in spleen and lymph nodes (Elomaa *et al.*, 1995).

Determination of the bacteria-binding motif in MARCO (Paper III)

The bacteria-binding region of MARCO is located in domain V. This observation was done earlier, but it was concluded from a limited set of constructs (Elomaa *et al.*, 1998). We decided to try to confirm this finding and narrow the bacteria-binding region down to find possible critical amino acids involved. We used truncated constructs of both human and mouse MARCO that did not contain domain V or contained 22 amino acids of the most proximal part of domain V. When transfected into CHO cells, it turned out that the latter form exhibits bacteria binding activity while the former one did not. In addition, two human constructs were used lacking the 89 and 78 most C-terminal residues of domain V, respectively. Since the latter one had no significant bacteria-binding activity, we concluded that the 11-residue segment RGRAEVYYSGT only present in the former construct is important for bacterial binding. These data confirm previous findings, and demonstrate that the predominant bacteria-binding region in MARCO is located in the proximal part of domain V.

The ligand-binding domain of the structurally related receptor SR-A has been localized to the distal part of the collagenous domain where a lysine-rich cluster has been reported to play a critical role (Acton *et al.*, 1993; Doi *et al.*, 1993). A similar cluster of lysine residues exists also in the corresponding position of MARCO. Therefore, we considered the possibility that the major binding site is after all in the collagenous domain, but that these 22 amino acids of domain V are necessary for correct folding of the bacteria-binding site. However, this hypothesis turned out to be wrong, as shown using variants of MARCO containing deletions in the various part of the collagenous domain. Thus, we can conclude that the two related scavenger receptors, MARCO and SR-A, are not equipped with identical ligand-binding properties. The bacteria-binding activity of domain V of MARCO is the first specific function identified for the SRCR domain of scavenger receptors. It is not known whether the SRCR domain of SR-A has the ability of binding bacteria. Still, there is evidence for differential binding of LPS to the SR-AI and AII (Ashkenas *et al.*, 1993), suggesting that the SRCR domain is involved in the binding of LPS. A secreted form of bovine SR-AI binds to gram-positive bacteria

and recognizes LTA, but a similar form of SR-AII has not been tested in these experiments (Dunne *et al*., 1994).

Having demonstrated the importance of the proximal part of domain V for the bacteria-binding activity of MARCO, we carried out experiments which revealed a crucial role of the arginines of this segment in bacterial binding. We were able to identify the minimal requirements for the high-affinity binding, at least in the case of the truncated forms of MARCO. These studies indicated that a closely spaced arginine-doublet is sufficient to provide the bacterial binding. One evidence for this conclusion came from the studies with the truncated human construct h-431, and a variant of h-431, where serine-423 was substituted by an arginine. Thus, while h-431 more or less lacked bacteria-binding activity, the mutated form had approximately similar activity as the full-length MARCO (see figure 8). It is evident that the motif RVR is responsible for the dramatic increase of bacteria-binding activity. In another set of experiments, we could demonstrate that bacterial binding remains in a construct containing a similar motif RGR, while the activity is abolished when the motif is deleted. To explore whether the interactions between the MARCO forms and bacteria are simply due to a charge interaction between the positively charged arginines and the negative surface of the bacteria, we tested the effects of some negatively charged compounds on the binding. The binding was completely abolished when incubated with poly(I), but not when incubated with poly(C) or heparin. Of these compounds only poly(I) is a ligand of SRs. This finding suggests that the mutant still exhibits scavenger receptor-type binding specificity, despite lacking most of domain V.

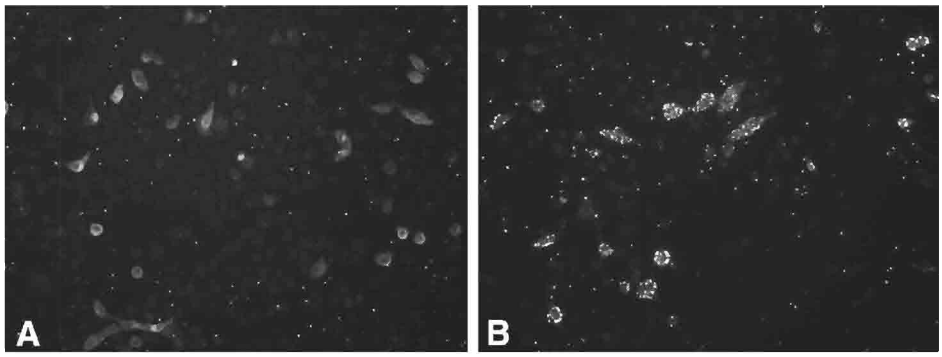

Figure 8. A single amino-acid substitution dramatically increases the bacteria-binding activity of h-431. CHO transfectants expressing h-431 lacks bacteria-binding activity (A). Substitution of serine-423 by an arginine generates a form, h-431(S423R), which binds bacteria strongly (B). h-431 has one arginine residue within its domain V segment, whereas h-431(S423R) has a closely spaced arginine doublet.

Even if the RXR motifs are essential for the bacterial binding in the truncated MARCO forms, our impression is that determination of the crystal structure of domain V with a ligand is required to unequivocally identify the ligand-binding site. The crystal structure of the SRCR domain of the MAC-2 binding protein (Hohenester *et al.*, 1999) has been used to model the structure of MARCO domain V (E. Morgunova unpublished observation). The two related domains have 46% identity. The modelling suggests that there is a cluster of arginines containing the first arginines of the domain and three arginines within residues 460-468. Interestingly, this latter segment also contains an RGR motif. Proteins with SRCR domains belongs to an ancient highly conserved family (Freeman *et al.*, 1990). This family includes a number of leukocyte antigens, for example, CD5 and CD6, but also proteins from primitive organisms, such as sea urchin speract receptor (Resnick *et al.*, 1994). To date, there is very little data about the ligands of the SRCR domains. Until recently, only the ligand for the SRCR domain of CD6, a T- and B-cell surface protein, was known. It binds ALCAM (Whitney *et al.*, 1995), which is a membrane protein of thymic epithelial cells and activated leucocytes (Bowen *et al.*, 1995). This year, a protein fragment of salivary agglutinin (gp-340/DMBT1) composed of 13 highly homologous SRCR domains was reported to bind bacteria, such as *Streptococcus mutans* (Bikker *et al.*, 2002). Salivary agglutinin has been implicated in the

oral clearance of microorganisms because of its bacteria-agglutinating properties (Ericson and Rundegren, 1983). Several consensus-based peptides of the SRCR domains and segments between them were synthesized, and their bacteria-binding activity were tested. Only one of the peptides was able to bind bacteria. Strikingly, the first 11 residues of this peptide exhibit 55% homology with the 11-residue bacteria-binding segment of MARCO identified in our first assays. The salivary agglutinin peptide does not include a RXR motif but a QXR motif, suggesting that both RXR and QXR motifs can play a role in bacterial binding.

Production and characterization of a recombinant soluble MARCO (Paper IV)

To produce a soluble form of MARCO (sMARCO) a construct where the intracellular and transmembrane domains of MARCO were replaced by a fragment encoding the mouse immunoglobulin kappa (Igκ) chain leader sequence was made. This modification allowed secretion of sMARCO into the culture medium. In addition, the signal sequence was followed by a polyhistidine tag to facilitate the purification. The construct was transfected into 293/EBNA cells. This cell line constitutively expresses the EBNA-1 protein of Epstein-Barr virus, thus allowing episomal replication of a vector containing the EBV origin of replication. Following screening of transfected 293/EBNA cells, several stable sMARCO-producing clones were obtained. sMARCO was then purified on Ni-NTA resins. Previously, we attempted to produce an untagged version of sMARCO, but these experiments were not succesful. It was found that MARCO is quite a "sticky" protein, because it binds to the plastic tube during storagement at 4°C. Thus, it is possible that the untagged sMARCO became bound to the chromatographic matrices during the purification process. This speculation is supported by the finding that MARCO binds unopsonized environmental particles (Palecanda *et al.*, 1999). Therefore, we developed a one step purification protocol to obtain better yields of the sMARCO protein. Purified sMARCO migrated as two forms with appearent molecular masses of 65 and 72kDa, respectively, on SDS-PAGE. This might be due to differences in glycosylation or hydroxylation, as well as to proteolytic processing. N-glycosidase F-treatment indicated

that the 72 kDa form of sMARCO has two N-linked oligosaccharide side chains, and that the 65 kDa form has one. Thus, it seems that both putative glycosylation sites are used in MARCO. When sMARCO was analyzed during unreduced conditions, two bands with approximately molecular masses around 220 kDa and 70 kDa, could be distinguished. This suggest that a fraction of sMARCO has formed a covalently-associated triple-helical molecule. Circular dichroism analysis were used to confirm that sMARCO molecules have assembled into a triple helix. Indeed, the purified sMARCO produced a spectrum typical for triple-helical collagens (Piez and Sherman, 1970). The melting temperature was determined to 44°C. Similar melting temperatures have been measured for recombinant type X collagen and type X/II chimeric collagens produced in 293 cells (Middleton and Bulleid, 1993; Schulte *et al.*, 1998). sMARCO is resistant to trypsin digestion, again demonstrating that a major part of the purified protein has folded into a triple helix. The trypsin digestion increases the amount of the 65 kDa form, whereas the amount of the otherwise predominant 72 kDa form decreases.

Rotary shadowing electron microscopy analysis revealed dumbbell-shaped particles with globular domains at both ends interlinked by a rod-like domain of 82.7 nm average length. This length corresponds well with the calculated length of an 89 Gly-X-Y-repeats-containing triple helix (Traub and Piez, 1971). The globular ends correspond to SRCR and spacer domains, but it is not possible to distinguish these domains morphologically. About 50 % of all molecules are associated by the globes, forming linear dimers, trimers or even starlike complexes. If this is a real property of MARCO one can speculate that MARCO molecules adhere to each other via SRCR domain to form a tight network of MARCO-positive cells in organs such as spleen, where the marginal zone macrophages filter the passing bloodstream. It is noteworthy that the soluble form of SR-A was not reported to form higher order oligomers (Resnick *et al.*, 1993). Both MARCO and SR-A are structurally related to the defense collagens or collectins (see "*pattern-recognition receptors*"), which all appear to participate in host defense. All the collectins have a relatively short collagenous domain and form higher order oligomers in which multiple collagenous stalks support globular head groups (Thiel and Reid, 1989). We have also produced a soluble SRCR domain (domain V), referred to recV, to explore how important the trimerization is for the bacteria binding capacity. Our

earlier studies with transmembrane MARCO have indicated a crucial role for domain V for this function (Brannstrom *et al*., 2002; Elomaa *et al*., 1998). Here we demonstrate that sMARCO binds bacteria avidly, but rec V showed almost no binding ability, indicating the importance of the trimerization for this function of MARCO. sMARCO also exhibits other binding characteristics typical to scavenger receptors, because it binds poly(I) but not the control polyanion heparin.

Since it is not known whether MARCO binds living bacteria we decided to test three different bacterial strains, wild-type *E .coli* K12, *E .Coli* strain HB101, and *S. typhimurium* LB5010, which all were FITC-labeled. Wild-type *E. coli* K12 expresses the smooth form of LPS, whereas *E. coli* HB101 and *S. typhimurium* LB5010 express the rough form (Bullas and Ryu, 1983; Goldberg *et al*., 1992). MARCO binds all strains, but it binds the two strains with rough forms of LPS better than the strain with the smooth form. This finding is well in line with the knowledge that pathogenic bacteria, which express smooth LPS, are more resistant to the clearance by phagocytes compared to rough strains lacking the O-side chains (Alexander and Rietschel, 2001). We also demonstrated that MARCO binds LPS using two different assays. This means that MARCO has at least another bacterial ligand, since it binds both gram-negative and gram-positive bacteria (Elomaa *et al*., 1995).

CONCLUSIONS

This work has provided new information about the structure and chromosomal locations of the human and mouse MARCO genes, as well as about the properties of the MARCO protein. Our studies have clearly improved our understanding about the functions of MARCO, and it also provides new directions for future research.

The studies on the human and mouse MARCO genes were important for several reasons. First, the results will facilitate studies on the potential involvement of MARCO and its gene in diseases affecting the host defense. However, at present no such diseases have been localized to the MARCO gene locus on chromosome 2. Second, knowledge about the nucleotide sequences of the genes may help to identify transcription factors that regulate the expression of macrophage-specific proteins such as MARCO. Both human and mouse genes have a TATA box, which is common for genes expressed in a cell-specific manner. Interestingly, both genes have a potential binding site for PU.1, a transcription factor that is a candidate for mediating macrophage-specific gene expression (Klemsz *et al*., 1990). Furthermore, the mouse gene has a sequence that could be a target for the transcription factor NFκB, which is involved in the expression of several genes in response to LPS. In ongoing experiments, transgenic mice are being generated using constructs containing different lengths of the upstream region of the gene, to determine the promotor region and other elements necessary for MARCO expression. We have also determined the transcription initiation site of the mouse MARCO gene to elucidate the sequence of the 5´end of the mRNA and the translation initiation site. The results suggest that the major form of MARCO has only a 20 amino residue long cytoplasmic domain. It remains to be seen whether this segment, or the longer version of the cytoplasmic domain, has any signaling function of its own. Finally, the characterization of the mouse gene has facilitated generation of MARCO-deficient mice that can be used to study the biological role of MARCO.

Studies on the functions of MARCO indicated that MARCO has at least two different properties that may be of importance for its function in the innate immune system. One can easily imagine that the MARCO-induced cell shape changes could be of importance for enlarging the cell surface of the MARCO-expressing macrophages to

facilitate the capture of foreign particles, such as pathogens. Cell shape changes are also necessary for efficient phagocytosis of large particles, and the long extensions induced by MARCO could facilitate this process. The signalling events involved in these dramatic morphological changes remain to be elucidated. It is true that all the experiments concerning the morphological activity of MARCO have been done in non-macrophage-cell lines, and therefore the impact of MARCO on the macrophage morphology still needs further investigation. In any case, it is of interest to note here that the MARCO-expressing marginal zone macrophages are large cells with long processes (Kraal, 1992). Preliminary data show that peritoneal macrophages isolated from MARCO-deficient mice are spreading less than macrophages of the control animals. When staining for marginal zone macrophages of the spleen, the zone seems more disorganized in the knockout mice compared with the wild-type mice (Yunying Chen, personal communication).

Previous work (Elomaa *et al.*, 1998) provided the first evidence that the SRCR domain is the bacteria-binding domain of MARCO. This was an unexpected finding since several reports had indicated that the ligand-binding domain of the structurally related SR-A resides in the collagenous domain (Andersson and Freeman, 1998; Doi *et al.*, 1993). In our studies extensive efforts were first made to confirm the finding that SRCR domain is crucial for the bacteria-binding, and then we tried to elucidate what are the critical elements for the binding. It was necessary to confirm the findings of the first study, because that work had been done with a limited set of constructs. For example, no constructs with internal deletions had been used. Having convincingly demonstrated the importance of the SRCR domain for the bacteria-binding activity, we started to mutate residues potentially important for the bacteria-binding function. We intended to study the effects of these mutations for bacterial binding in the context of the full-length MARCO. However, most of the mutated versions failed to be expressed on the cell surface, and therefore this approach was not pursued. Instead, we focused on analyzing truncated MARCO variants containing only portions of the SRCR domain. The purpose was to understand, with the help of these constructs, that what are the minimal requirements for high-affinity bacterial binding. This approach led to the finding that a motif containing an

arginine-doublet, RXR, is needed for this function, at least in the case of the truncated MARCO variants.

It is true that, based on these experiments, we cannot claim that a similar linear motif is the bacteria-binding motif in the full-length MARCO. However, our results indicate that this short motif, containing two positively charged amino acids, is enough to provide avid bacterial binding, at least when present in "a trimeric form". It is possible that in case of an intact domain V, arginines that are not necessarily next to each other in the linear sequence cluster together, and that this cluster facilitates high-affinity bacteria-binding. Modelling of MARCO indeed indicates that this is a possible scenario (E. Morgunova unpublished observation). Our understanding is that it is not possible to determine conclusively the residues involved in ligand binding by the mutagenesis/transfection approach, but that also other approaches will be needed.

The production of sMARCO made it possible to "view" MARCO and study its binding properties in a cell-free system. sMARCO was shown to have a triple-helical collagenous structure. The molecules were dumbbell-shaped particles with globular domains at both ends interlinked by a rod-like domain of 82.7 nm. Rotary shadowing also demonstrated that sMARCO molecules often associate to each other by the globular ends. Our binding studies indicated that MARCO binds both heat-killed and living bacteria, and that also LPS is a ligand of MARCO. The O-side chains of LPS were not found to be important for bacterial recognition by MARCO. It remains to be studied that what is the precise role of MARCO in the LPS-signalling. In one set of experiments, the importance of trimerization of MARCO for the binding activity was also demonstrated. Our plan is now to use sMARCO for searching other ligands of MARCO.

ACKNOWLEDGEMENTS

I would like to thank all the collegues, friends, and family who made this thesis possible by being so supportive and encouraging during these years. In particular, I would like to thank the following persons who have contributed to the work in different ways.

First of all, my supervisor Karl Tryggvason, for your faith in me and the project, without you this thesis had never been done.

My co-supervisor Timo Pikkarainen, for taking your time to discuss with me, for giving me experienced advice in the lab, and for all help with the manuscripts and this thesis. Finally, for always being a nice company in the lab.

My former co-supervisor Outi Elomaa, for introducing me into the lab, for always having time for me, and for being a good friend.

All former and present members of the MARCO team and other collaborators, for fruitful collaborations.

Birgitta Agerberth, för att alltid ha tid att diskutera mina projekt och för alla uppmuntrande ord.

Annika Scheynius och ditt lab, för att ni hade både labplats och engagemang över till mig när jag behövde lära mig immunohistokemi.

Marko, för all hjälp med datorer och andra maskiner, för alla antikroppar och för ditt goda humör.

Annemay och Pekka, för att ni tog er tid att läsa manuskriptet och för era värdefulla kommentarer.

Ari, för all hjälp med datorer.

Kerstin, för att alltid ge mig ett vänligt bemötande när jag kommer med alla mina papper.

Jill, för att vara en nära vän och för allt stöd och engagemang i stort och smått på och utanför lab.

Jamshid, Bernd, Antti, and Pekka, for nice company in the office, and for always being friendly and supportive when I needed it.

Annemay, Olga, Tiina, Li, Ulla and Margareta for all nice lunches and chats.

Åsa och Cicci för att ni tog så väl hand om mig när jag flyttade till Stockholm och för alla våra långa fikastunder.

ACKNOWLEDGEMENTS

Bernt-Åke och Jessica, för ert trevliga sällskap och goda mat, för att ni ställer upp som barnvakt och tar så väl hand om Inez.

Mamma och pappa, för att ni finns och alltid ställer upp och för att ni tror på mig även när jag inte gör det själv.

Min älskade lilla familj, Andreas och Inez för att ni finns och ständigt påminner mig om att det finns ett liv utanför lab.

This work was funded by the Swedish Natural Science Research Council.

REFERENCES

Acton, S., Resnick, D., Freeman, M., Ekkel, Y., Ashkenas, J. and Krieger, M. (1993) The collagenous domains of macrophage scavenger receptors and complement component C1q mediate their similar, but not identical, binding specificities for polyanionic ligands. *J Biol Chem*, **268**, 3530-3537.

Aftring, R.P. and Freeman, M.W. (1995) Structure of the murine macrophage scavenger receptor gene and evaluation of sequences that regulate expression in the macrophage cell line, P388D. *J Lipid Res*, **36**, 1305-1314.

Alexander, C. and Rietschel, E.T. (2001) Bacterial lipopolysaccharides and innate immunity. *J Endotoxin Res*, **7**, 167-202.

Andersson, L. and Freeman, M.W. (1998) Functional changes in scavenger receptor binding conformation are induced by charge mutants spanning the entire collagen domain. *J Biol Chem*, **273**, 19592-19601.

Antic, A., Dzenko, K.A. and Pachter, J.S. (2000) Engagement of the scavenger receptor is not responsible for beta- amyloid stimulation of monocytes to a neurocytopathic state. *Exp Neurol*, **161**, 96-101.

Ashkenas, J., Penman, M., Vasile, E., Acton, S., Freeman, M. and Krieger, M. (1993) Structures and high and low affinity ligand binding properties of murine type I and type II macrophage scavenger receptors. *J Lipid Res*, **34**, 983-1000.

Bell, M.D., Lopez-Gonzalez, R., Lawson, L., Hughes, D., Fraser, I., Gordon, S. and Perry, V.H. (1994) Upregulation of the macrophage scavenger receptor in response to different forms of injury in the CNS. *J Neurocytol*, **23**, 605-613.

Belvin, M.P. and Anderson, K.V. (1996) A conserved signaling pathway: the Drosophila toll-dorsal pathway. *Annu Rev Cell Dev Biol*, **12**, 393-416.

Bikker, F.J., Ligtenberg, A.J., Nazmi, K., Veerman, E.C., Van 't Hof, W., Bolscher, J.G., Poustka, A., Nieuw Amerongen, A.V. and Mollenhauer, J. (2002) Identification of the bacteria-binding peptide domain on salivary agglutinin (gp-340/DMBT1), a member of the scavenger receptor-cysteine rich superfamily. *J Biol Chem*, **5**, 5.

Bowen, M.A., Patel, D.D., Li, X., Modrell, B., Malacko, A.R., Wang, W.C., Marquardt, H., Neubauer, M., Pesando, J.M., Francke, U. and et al. (1995) Cloning, mapping, and characterization of activated leukocyte-cell adhesion molecule (ALCAM), a CD6 ligand. *J Exp Med*, **181**, 2213-2220.

Brannstrom, A., Sankala, M., Tryggvason, K. and Pikkarainen, T. (2002) Arginine residues in domain V have a central role for bacteria-binding activity of macrophage scavenger receptor MARCO. *Biochem Biophys Res Commun*, **290**, 1462-1469.

Brightbill, H.D., Libraty, D.H., Krutzik, S.R., Yang, R.B., Belisle, J.T., Bleharski, J.R., Maitland, M., Norgard, M.V., Plevy, S.E., Smale, S.T., Brennan, P.J., Bloom, B.R., Godowski, P.J. and Modlin, R.L. (1999) Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. *Science*, **285**, 732-736.

Bullas, L.R. and Ryu, J.I. (1983) Salmonella typhimurium LT2 strains which are r- m+ for all three chromosomally located systems of DNA restriction and modification. *J Bacteriol*, **156**, 471-474.

REFERENCES

Burridge, K. and Chrzanowska-Wodnicka, M. (1996) Focal adhesions, contractility, and signaling. *Annu Rev Cell Dev Biol*, **12**, 463-518.

Christie, R.H., Freeman, M. and Hyman, B.T. (1996) Expression of the macrophage scavenger receptor, a multifunctional lipoprotein receptor, in microglia associated with senile plaques in Alzheimer's disease. *Am J Pathol*, **148**, 399-403.

Clemens, M.J. (1997) PKR--a protein kinase regulated by double-stranded RNA. *Int J Biochem Cell Biol*, **29**, 945-949.

de Villiers, W.J., Fraser, I.P., Hughes, D.A., Doyle, A.G. and Gordon, S. (1994) Macrophage-colony-stimulating factor selectively enhances macrophage scavenger receptor expression and function. *J Exp Med*, **180**, 705-709.

de Winther, M.P., Gijbels, M.J., van Dijk, K.W., van Gorp, P.J., suzuki, H., Kodama, T., Frants, R.R., Havekes, L.M. and Hofker, M.H. (1999a) Scavenger receptor deficiency leads to more complex atherosclerotic lesions in APOE3Leiden transgenic mice. *Atherosclerosis*, **144**, 315-321.

de Winther, M.P., van Dijk, K.W., van Vlijmen, B.J., Gijbels, M.J., Heus, J.J., Wijers, E.R., van den Bos, A.C., Breuer, M., Frants, R.R., Havekes, L.M. and Hofker, M.H. (1999b) Macrophage specific overexpression of the human macrophage scavenger receptor in transgenic mice, using a 180-kb yeast artificial chromosome, leads to enhanced foam cell formation of isolated peritoneal macrophages. *Atherosclerosis*, **147**, 339-347.

Dijkstra, C.D., Van Vliet, E., Dopp, E.A., van der Lelij, A.A. and Kraal, G. (1985) Marginal zone macrophages identified by a monoclonal antibody: characterization of immuno- and enzyme-histochemical properties and functional capacities. *Immunology*, **55**, 23-30.

Doi, T., Higashino, K., Kurihara, Y., Wada, Y., Miyazaki, T., Nakamura, H., Uesugi, S., Imanishi, T., Kawabe, Y., Itakura, H. and et al. (1993) Charged collagen structure mediates the recognition of negatively charged macromolecules by macrophage scavenger receptors. *J Biol Chem*, **268**, 2126-2133.

Doi, T., Kurasawa, M., Higashino, K., Imanishi, T., Mori, T., Naito, M., Takahashi, K., Kawabe, Y., Wada, Y., Matsumoto, A. and et al. (1994) The histidine interruption of an alpha-helical coiled coil allosterically mediates a pH-dependent ligand dissociation from macrophage scavenger receptors. *J Biol Chem*, **269**, 25598-25604.

Domanico, S.Z., Pelletier, A.J., Havran, W.L. and Quaranta, V. (1997) Integrin alpha 6A beta 1 induces CD81-dependent cell motility without engaging the extracellular matrix migration substrate. *Mol Biol Cell*, **8**, 2253-2265.

Drickamer, K. and McCreary, V. (1987) Exon structure of a mannose-binding protein gene reflects its evolutionary relationship to the asialoglycoprotein receptor and nonfibrillar collagens. *J Biol Chem*, **262**, 2582-2589.

Dunne, D.W., Resnick, D., Greenberg, J., Krieger, M. and Joiner, K.A. (1994) The type I macrophage scavenger receptor binds to gram-positive bacteria and recognizes lipoteichoic acid. *Proc Natl Acad Sci U S A*, **91**, 1863-1867.

El Khoury, J., Hickman, S.E., Thomas, C.A., Cao, L., Silverstein, S.C. and Loike, J.D. (1996) Scavenger receptor-mediated adhesion of microglia to beta-amyloid fibrils. *Nature*, **382**, 716-719.

REFERENCES

Elomaa, O. (1996) *Novel Type of Macrophage Receptor with Collagenous Structure (MARCO).* Acta University of Oulu, Oulu.

Elomaa, O., Kangas, M., Sahlberg, C., Tuukkanen, J., Sormunen, R., Liakka, A., Thesleff, I., Kraal, G. and Tryggvason, K. (1995) Cloning of a novel bacteria-binding receptor structurally related to scavenger receptors and expressed in a subset of macrophages. *Cell*, **80**, 603-609.

Elomaa, O., Sankala, M., Pikkarainen, T., Bergmann, U., Tuuttila, A., Raatikainen-Ahokas, A., Sariola, H. and Tryggvason, K. (1998) Structure of the human macrophage MARCO receptor and characterization of its bacteria-binding region. *J Biol Chem*, **273**, 4530-4538.

Elshourbagy, N.A., Li, X., Terrett, J., Vanhorn, S., Gross, M.S., Adamou, J.E., Anderson, K.M., Webb, C.L. and Lysko, P.G. (2000) Molecular characterization of a human scavenger receptor, human MARCO. *Eur J Biochem*, **267**, 919-926.

Emi, M., Asaoka, H., Matsumoto, A., Itakura, H., Kurihara, Y., Wada, Y., Kanamori, H., Yazaki, Y., Takahashi, E., Lepert, M. and et al. (1993) Structure, organization, and chromosomal mapping of the human macrophage scavenger receptor gene. *J Biol Chem*, **268**, 2120-2125.

Ericson, T. and Rundegren, J. (1983) Characterization of a salivary agglutinin reacting with a serotype c strain of Streptococcus mutans. *Eur J Biochem*, **133**, 255-261.

Feinberg, A.P. and Vogelstein, B. (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem*, **132**, 6-13.

Ferrero, E., Jiao, D., Tsuberi, B.Z., Tesio, L., Rong, G.W., Haziot, A. and Goyert, S.M. (1993) Transgenic mice expressing human CD14 are hypersensitive to lipopolysaccharide. *Proc Natl Acad Sci U S A*, **90**, 2380-2384.

Fitzgerald, M.L., Moore, K.J., Freeman, M.W. and Reed, G.L. (2000) Lipopolysaccharide induces scavenger receptor A expression in mouse macrophages: a divergent response relative to human THP-1 monocyte/macrophages. *J Immunol*, **164**, 2692-2700.

Fraser, I., Hughes, D. and Gordon, S. (1993) Divalent cation-independent macrophage adhesion inhibited by monoclonal antibody to murine scavenger receptor. *Nature*, **364**, 343-346.

Freeman, M., Ashkenas, J., Rees, D.J., Kingsley, D.M., Copeland, N.G., Jenkins, N.A. and Krieger, M. (1990) An ancient, highly conserved family of cysteine-rich protein domains revealed by cloning type I and type II murine macrophage scavenger receptors. *Proc Natl Acad Sci U S A*, **87**, 8810-8814.

Geng, Y.J. and Hansson, G.K. (1995) High endothelial cells of postcapillary venules express the scavenger receptor in human peripheral lymph nodes. *Scand J Immunol*, **42**, 289-296.

Goldberg, J.B., Hatano, K., Meluleni, G.S. and Pier, G.B. (1992) Cloning and surface expression of Pseudomonas aeruginosa O antigen in Escherichia coli. *Proc Natl Acad Sci U S A*, **89**, 10716-10720.

Goldstein, J.L., Ho, Y.K., Basu, S.K. and Brown, M.S. (1979) Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc Natl Acad Sci U S A*, **76**, 333-337.

REFERENCES

Gottar, M., Gobert, V., Michel, T., Belvin, M., Duyk, G., Hoffmann, J.A., Ferrandon, D. and Royet, J. (2002) The Drosophila immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein. *Nature*, **416**, 640-644.

Gough, P.J., Greaves, D.R. and Gordon, S. (1998) A naturally occurring isoform of the human macrophage scavenger receptor (SR-A) gene generated by alternative splicing blocks modified LDL uptake. *J Lipid Res*, **39**, 531-543.

Hall, A. (1998) Rho GTPases and the actin cytoskeleton. *Science*, **279**, 509-514.

Hampton, R.Y., Golenbock, D.T., Penman, M., Krieger, M. and Raetz, C.R. (1991) Recognition and plasma clearance of endotoxin by scavenger receptors. *Nature*, **352**, 342-344.

Haworth, R., Platt, N., Keshav, S., Hughes, D., Darley, E., Suzuki, H., Kurihara, Y., Kodama, T. and Gordon, S. (1997) The macrophage scavenger receptor type A is expressed by activated macrophages and protects the host against lethal endotoxic shock. *J Exp Med*, **186**, 1431-1439.

Haziot, A., Ferrero, E., Kontgen, F., Hijiya, N., Yamamoto, S., Silver, J., Stewart, C.L. and Goyert, S.M. (1996) Resistance to endotoxin shock and reduced dissemination of gram- negative bacteria in CD14-deficient mice. *Immunity*, **4**, 407-414.

Hohenester, E., Sasaki, T. and Timpl, R. (1999) Crystal structure of a scavenger receptor cysteine-rich domain sheds light on an ancient superfamily. *Nat Struct Biol*, **6**, 228-232.

Horvai, A., Palinski, W., Wu, H., Moulton, K.S., Kalla, K. and Glass, C.K. (1995) Scavenger receptor A gene regulatory elements target gene expression to macrophages and to foam cells of atherosclerotic lesions. *Proc Natl Acad Sci U S A*, **92**, 5391-5395.

Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K. and Akira, S. (1999) Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol*, **162**, 3749-3752.

Huang, F., Buttini, M., Wyss-Coray, T., McConlogue, L., Kodama, T., Pitas, R.E. and Mucke, L. (1999) Elimination of the class A scavenger receptor does not affect amyloid plaque formation or neurodegeneration in transgenic mice expressing human amyloid protein precursors. *Am J Pathol*, **155**, 1741-1747.

Hughes, D.A., Fraser, I.P. and Gordon, S. (1995) Murine macrophage scavenger receptor: in vivo expression and function as receptor for macrophage adhesion in lymphoid and non-lymphoid organs. *Eur J Immunol*, **25**, 466-473.

Ito, S., Naito, M., Kobayashi, Y., Takatsuka, H., Jiang, S., Usuda, H., Umezu, H., Hasegawa, G., Arakawa, M., Shultz, L.D., Elomaa, O. and Tryggvason, K. (1999) Roles of a macrophage receptor with collagenous structure (MARCO) in host defense and heterogeneity of splenic marginal zone macrophages. *Arch Histol Cytol*, **62**, 83-95.

Kataoka, H., Kume, N., Miyamoto, S., Minami, M., Murase, T., Sawamura, T., Masaki, T., Hashimoto, N. and Kita, T. (2000) Biosynthesis and post-translational processing of lectin-like oxidized low density lipoprotein receptor-1 (LOX-1). N-

linked glycosylation affects cell-surface expression and ligand binding. *J Biol Chem*, **275**, 6573-6579.

Khush, R.S., Leulier, F. and Lemaitre, B. (2001) Drosophila immunity: two paths to NF-kappaB. *Trends Immunol*, **22**, 260-264.

Klemsz, M.J., McKercher, S.R., Celada, A., Van Beveren, C. and Maki, R.A. (1990) The macrophage and B cell-specific transcription factor PU.1 is related to the ets oncogene. *Cell*, **61**, 113-124.

Kobayashi, Y., Miyaji, C., Watanabe, H., Umezu, H., Hasegawa, G., Abo, T., Arakawa, M., Kamata, N., Suzuki, H., Kodama, T. and Naito, M. (2000) Role of macrophage scavenger receptor in endotoxin shock. *J Pathol*, **192**, 263-272.

Kobzik, L. (1995) Lung macrophage uptake of unopsonized environmental particulates. Role of scavenger-type receptors. *J Immunol*, **155**, 367-376.

Kodama, T., Freeman, M., Rohrer, L., Zabrecky, J., Matsudaira, P. and Krieger, M. (1990) Type I macrophage scavenger receptor contains alpha-helical and collagen-like coiled coils. *Nature*, **343**, 531-535.

Kodama, T., Reddy, P., Kishimoto, C. and Krieger, M. (1988) Purification and characterization of a bovine acetyl low density lipoprotein receptor. *Proc Natl Acad Sci U S A*, **85**, 9238-9242.

Kopp, E.B. and Medzhitov, R. (1999) The Toll-receptor family and control of innate immunity. *Curr Opin Immunol*, **11**, 13-18.

Kozak, M. (1989) The scanning model for translation: an update. *J Cell Biol*, **108**, 229-241.

Kraal, G. (1992) Cells in the marginal zone of the spleen. *Int Rev Cytol*, **132**, 31-74.

Kraal, G. and Janse, M. (1986) Marginal metallophilic cells of the mouse spleen identified by a monoclonal antibody. *Immunology*, **58**, 665-669.

Krieger, M. and Herz, J. (1994) Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). *Annu Rev Biochem*, **63**, 601-637.

Kurt-Jones, E.A., Popova, L., Kwinn, L., Haynes, L.M., Jones, L.P., Tripp, R.A., Walsh, E.E., Freeman, M.W., Golenbock, D.T., Anderson, L.J. and Finberg, R.W. (2000) Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat Immunol*, **1**, 398-401.

Lee, S.J., Evers, S., Roeder, D., Parlow, A.F., Risteli, J., Risteli, L., Lee, Y.C., Feizi, T., Langen, H. and Nussenzweig, M.C. (2002) Mannose receptor-mediated regulation of serum glycoprotein homeostasis. *Science*, **295**, 1898-1901.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M. and Hoffmann, J.A. (1996) The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell*, **86**, 973-983.

Mato, M., Ookawara, S., Sakamoto, A., Aikawa, E., Ogawa, T., Mitsuhashi, U., Masuzawa, T., Suzuki, H., Honda, M., Yazaki, Y., Watanabe, E., Luoma, J., Yla-Herttuala, S., Fraser, I., Gordon, S. and Kodama, T. (1996) Involvement of specific macrophage-lineage cells surrounding arterioles in barrier and scavenger function in brain cortex. *Proc Natl Acad Sci U S A*, **93**, 3269-3274.

Matsuda, Y. and Chapman, V.M. (1995) Application of fluorescence in situ hybridization in genome analysis of the mouse. *Electrophoresis*, **16**, 261-272.

REFERENCES

Matsuda, Y., Harada, Y.N., Natsuume-Sakai, S., Lee, K., Shiomi, T. and Chapman, V.M. (1992) Location of the mouse complement factor H gene (cfh) by FISH analysis and replication R-banding. *Cytogenet Cell Genet*, **61**, 282-285.

Matsumoto, A., Naito, M., Itakura, H., Ikemoto, S., Asaoka, H., Hayakawa, I., Kanamori, H., Aburatani, H., Takaku, F., Suzuki, H. and et al. (1990) Human macrophage scavenger receptors: primary structure, expression, and localization in atherosclerotic lesions. *Proc Natl Acad Sci U S A*, **87**, 9133-9137.

McKercher, S.R., Torbett, B.E., Anderson, K.L., Henkel, G.W., Vestal, D.J., Baribault, H., Klemsz, M., Feeney, A.J., Wu, G.E., Paige, C.J. and Maki, R.A. (1996) Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *Embo J*, **15**, 5647-5658.

Medzhitov, R. (2001) Toll-like receptors and innate immunity. *Nat Rev Immunol*, **1**, 135-145.

Medzhitov, R., Preston-Hurlburt, P. and Janeway, C.A., Jr. (1997) A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature*, **388**, 394-397.

Michel, T., Reichhart, J.M., Hoffmann, J.A. and Royet, J. (2001) Drosophila Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. *Nature*, **414**, 756-759.

Middleton, R.B. and Bulleid, N.J. (1993) Reconstitution of the folding pathway of collagen in a cell-free system: formation of correctly aligned and hydroxylated triple helices. *Biochem J*, **296**, 511-517.

Morimoto, K., Wada, Y., Hinagata, J., Imanishi, T., Kodama, T. and Doi, T. (1999) VXFD in the cytoplasmic domain of macrophage scavenger receptors mediates their efficient internalization and cell-surface expression. *Biol Pharm Bull*, **22**, 1022-1026.

Moriwaki, H., Kume, N., Kataoka, H., Murase, T., Nishi, E., Sawamura, T., Masaki, T. and Kita, T. (1998) Expression of lectin-like oxidized low density lipoprotein receptor-1 in human and murine macrophages: upregulated expression by TNF-alpha. *FEBS Lett*, **440**, 29-32.

Moulton, K.S., Semple, K., Wu, H. and Glass, C.K. (1994) Cell-specific expression of the macrophage scavenger receptor gene is dependent on PU.1 and a composite AP-1/ets motif. *Mol Cell Biol*, **14**, 4408-4418.

Moulton, K.S., Wu, H., Barnett, J., Parthasarathy, S. and Glass, C.K. (1992) Regulated expression of the human acetylated low density lipoprotein receptor gene and isolation of promoter sequences. *Proc Natl Acad Sci U S A*, **89**, 8102-8106.

Nagai, Y., Akashi, S., Nagafuku, M., Ogata, M., Iwakura, Y., Akira, S., Kitamura, T., Kosugi, A., Kimoto, M. and Miyake, K. (2002) Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nat Immunol*, **3**, 667-672.

Nagase, M., Abe, J., Takahashi, K., Ando, J., Hirose, S. and Fujita, T. (1998) Genomic organization and regulation of expression of the lectin-like oxidized low-density lipoprotein receptor (LOX-1) gene. *J Biol Chem*, **273**, 33702-33707.

Naito, M., Kodama, T., Matsumoto, A., Doi, T. and Takahashi, K. (1991) Tissue distribution, intracellular localization, and in vitro expression of bovine macrophage scavenger receptors. *Am J Pathol*, **139**, 1411-1423.

Naito, M., Umeda, S., Takahashi, K. and Shultz, L.D. (1997) Macrophage differentiation and granulomatous inflammation in osteopetrotic mice (op/op) defective in the production of CSF-1. *Mol Reprod Dev*, **46**, 85-91.

Nakamura, K., Funakoshi, H., Miyamoto, K., Tokunaga, F. and Nakamura, T. (2001) Molecular cloning and functional characterization of a human scavenger receptor with C-type lectin (SRCL), a novel member of a scavenger receptor family. *Biochem Biophys Res Commun*, **280**, 1028-1035.

Nicoletti, A., Caligiuri, G., Tornberg, I., Kodama, T., Stemme, S. and Hansson, G.K. (1999) The macrophage scavenger receptor type A directs modified proteins to antigen presentation. *Eur J Immunol*, **29**, 512-521.

Ohashi, K., Burkart, V., Flohe, S. and Kolb, H. (2000) Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *J Immunol*, **164**, 558-561.

Ohtani, K., Suzuki, Y., Eda, S., Kawai, T., Kase, T., Keshi, H., Sakai, Y., Fukuoh, A., Sakamoto, T., Itabe, H., Suzutani, T., Ogasawara, M., Yoshida, I. and Wakamiya, N. (2001) The membrane-type collectin CL-P1 is a scavenger receptor on vascular endothelial cells. *J Biol Chem*, **276**, 44222-44228.

Ozinsky, A., Underhill, D.M., Fontenot, J.D., Hajjar, A.M., Smith, K.D., Wilson, C.B., Schroeder, L. and Aderem, A. (2000) The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc Natl Acad Sci U S A*, **97**, 13766-13771.

Palecanda, A., Paulauskis, J., Al-Mutairi, E., Imrich, A., Qin, G., Suzuki, H., Kodama, T., Tryggvason, K., Koziel, H. and Kobzik, L. (1999) Role of the scavenger receptor MARCO in alveolar macrophage binding of unopsonized environmental particles. *J Exp Med*, **189**, 1497-1506.

Pearson, A.M. (1996) Scavenger receptors in innate immunity. *Curr Opin Immunol*, **8**, 20-28.

Pearson, A.M., Rich, A. and Krieger, M. (1993) Polynucleotide binding to macrophage scavenger receptors depends on the formation of base-quartet-stabilized four-stranded helices. *J Biol Chem*, **268**, 3546-3554.

Piez, K.A. and Sherman, M.R. (1970) Characterization of the product formed by renaturation of alpha 1-CB2, a small peptide from collagen. *Biochemistry*, **9**, 4129-4133.

Pikkarainen, T., Brannstrom, A. and Tryggvason, K. (1999) Expression of macrophage MARCO receptor induces formation of dendritic plasma membrane processes. *J Biol Chem*, **274**, 10975-10982.

Platt, N., da Silva, R.P. and Gordon, S. (1998) Recognizing death: the phagocytosis of apoptotic cells. *Trends Cell Biol*, **8**, 365-372.

Platt, N. and Gordon, S. (1998) Scavenger receptors: diverse activities and promiscuous binding of polyanionic ligands. *Chem Biol*, **5**, R193-203.

Platt, N., Suzuki, H., Kodama, T. and Gordon, S. (2000) Apoptotic thymocyte clearance in scavenger receptor class A-deficient mice is apparently normal. *J Immunol*, **164**, 4861-4867.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Huffel, C.V., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B.

and Beutler, B. (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science*, **282**, 2085-2088.

Qureshi, S.T., Lariviere, L., Leveque, G., Clermont, S., Moore, K.J., Gros, P. and Malo, D. (1999) Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *J Exp Med*, **189**, 615-625.

Ramet, M., Manfruelli, P., Pearson, A., Mathey-Prevot, B. and Ezekowitz, R.A. (2002) Functional genomic analysis of phagocytosis and identification of a Drosophila receptor for E. coli. *Nature*, **416**, 644-648.

Ramet, M., Pearson, A., Manfruelli, P., Li, X., Koziel, H., Gobel, V., Chung, E., Krieger, M. and Ezekowitz, R.A. (2001) Drosophila scavenger receptor CI is a pattern recognition receptor for bacteria. *Immunity*, **15**, 1027-1038.

Resnick, D., Chatterton, J.E., Schwartz, K., Slayter, H. and Krieger, M. (1996) Structures of class A macrophage scavenger receptors. Electron microscopic study of flexible, multidomain, fibrous proteins and determination of the disulfide bond pattern of the scavenger receptor cysteine-rich domain. *J Biol Chem*, **271**, 26924-26930.

Resnick, D., Freedman, N.J., Xu, S. and Krieger, M. (1993) Secreted extracellular domains of macrophage scavenger receptors form elongated trimers which specifically bind crocidolite asbestos. *J Biol Chem*, **268**, 3538-3545.

Resnick, D., Pearson, A. and Krieger, M. (1994) The SRCR superfamily: a family reminiscent of the Ig superfamily. *Trends Biochem Sci*, **19**, 5-8.

Sakaguchi, H., Takeya, M., Suzuki, H., Hakamata, H., Kodama, T., Horiuchi, S., Gordon, S., van der Laan, L.J., Kraal, G., Ishibashi, S., Kitamura, N. and Takahashi, K. (1998) Role of macrophage scavenger receptors in diet-induced atherosclerosis in mice. *Lab Invest*, **78**, 423-434.

Sambrook J., F.E.F., and Maniatis T. (1989) *Molecular cloning: a laboratory manual.* Cold Spring Harbour Laboratory Press, Cold Spring Harbour.

Schulte, S., Unger, C., Mo, J.A., Wendler, O., Bauer, E., Frischholz, S., von der Mark, K., Kalden, J.R., Holmdahl, R. and Burkhardt, H. (1998) Arthritis-related B cell epitopes in collagen II are conformation- dependent and sterically privileged in accessible sites of cartilage collagen fibrils. *J Biol Chem*, **273**, 1551-1561.

Seldin, M.F. (1994) Mouse chromosome 1. *Mamm Genome*, **5**, S1-21.

Shapiro, M.B. and Senapathy, P. (1987) RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. *Nucleic Acids Res*, **15**, 7155-7174.

Shibata, Y., Metzger, W.J. and Myrvik, Q.N. (1997) Chitin particle-induced cell-mediated immunity is inhibited by soluble mannan: mannose receptor-mediated phagocytosis initiates IL-12 production. *J Immunol*, **159**, 2462-2467.

Shimaoka, T., Kume, N., Minami, M., Hayashida, K., Sawamura, T., Kita, T. and Yonehara, S. (2001) LOX-1 supports adhesion of Gram-positive and Gram-negative bacteria. *J Immunol*, **166**, 5108-5114.

Singer, S.J. (1990) The structure and insertion of integral proteins in membranes. *Annu Rev Cell Biol*, **6**, 247-296.

Smith, D.B. and Johnson, K.S. (1988) Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. *Gene*, **67**, 31-40.

Stahl, P.D. and Ezekowitz, R.A. (1998) The mannose receptor is a pattern recognition receptor involved in host defense. *Curr Opin Immunol*, **10**, 50-55.

Suzuki, H., Kurihara, Y., Takeya, M., Kamada, N., Kataoka, M., Jishage, K., Ueda, O., Sakaguchi, H., Higashi, T., Suzuki, T., Takashima, Y., Kawabe, Y., Cynshi, O., Wada, Y., Honda, M., Kurihara, H., Aburatani, H., Doi, T., Matsumoto, A., Azuma, S., Noda, T., Toyoda, Y., Itakura, H., Yazaki, Y., Kodama, T. and et al. (1997) A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. *Nature*, **386**, 292-296.

Sweet, M.J. and Hume, D.A. (1996) Endotoxin signal transduction in macrophages. *J Leukoc Biol*, **60**, 8-26.

Takahashi, K., Naito, M., Shultz, L.D., Hayashi, S. and Nishikawa, S. (1993) Differentiation of dendritic cell populations in macrophage colony- stimulating factor-deficient mice homozygous for the osteopetrosis (op) mutation. *J Leukoc Biol*, **53**, 19-28.

Takahashi, K., Umeda, S., Shultz, L.D., Hayashi, S. and Nishikawa, S. (1994) Effects of macrophage colony-stimulating factor (M-CSF) on the development, differentiation, and maturation of marginal metallophilic macrophages and marginal zone macrophages in the spleen of osteopetrosis (op) mutant mice lacking functional M-CSF activity. *J Leukoc Biol*, **55**, 581-588.

Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K. and Akira, S. (1999) Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity*, **11**, 443-451.

Terpstra, V., van Amersfoort, E.S., van Velzen, A.G., Kuiper, J. and van Berkel, T.J. (2000) Hepatic and extrahepatic scavenger receptors: function in relation to disease. *Arterioscler Thromb Vasc Biol*, **20**, 1860-1872.

Thiel, S. and Reid, K.B. (1989) Structures and functions associated with the group of mammalian lectins containing collagen-like sequences. *FEBS Lett*, **250**, 78-84.

Thomas, C.A., Li, Y., Kodama, T., Suzuki, H., Silverstein, S.C. and El Khoury, J. (2000) Protection from lethal gram-positive infection by macrophage scavenger receptor-dependent phagocytosis. *J Exp Med*, **191**, 147-156.

Trask, B.J. (1991) DNA sequence localization in metaphase and interphase cells by fluorescence in situ hybridization. *Methods Cell Biol*, **35**, 3-35.

Traub, W. and Piez, K.A. (1971) The chemistry and structure of collagen. *Adv Protein Chem*, **25**, 243-352.

Underhill, D.M., Ozinsky, A., Hajjar, A.M., Stevens, A., Wilson, C.B., Bassetti, M. and Aderem, A. (1999) The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. *Nature*, **401**, 811-815.

Uthaisangsook, S., Day, N.K., Bahna, S.L., Good, R.A. and Haraguchi, S. (2002) Innate immunity and its role against infections. *Ann Allergy Asthma Immunol*, **88**, 253-264; quiz 265-256, 318.

Vabulas, R.M., Ahmad-Nejad, P., da Costa, C., Miethke, T., Kirschning, C.J., Hacker, H. and Wagner, H. (2001) Endocytosed HSP60s use toll-like receptor 2 (TLR2) and TLR4 to activate the toll/interleukin-1 receptor signaling pathway in innate immune cells. *J Biol Chem*, **276**, 31332-31339.

REFERENCES

van der Laan, L.J., Dopp, E.A., Haworth, R., Pikkarainen, T., Kangas, M., Elomaa, O., Dijkstra, C.D., Gordon, S., Tryggvason, K. and Kraal, G. (1999) Regulation and functional involvement of macrophage scavenger receptor MARCO in clearance of bacteria in vivo. *J Immunol*, **162**, 939-947.

van der Laan, L.J., Kangas, M., Dopp, E.A., Broug-Holub, E., Elomaa, O., Tryggvason, K. and Kraal, G. (1997) Macrophage scavenger receptor MARCO: in vitro and in vivo regulation and involvement in the anti-bacterial host defense. *Immunol Lett*, **57**, 203-208.

van Lenten, B.J. and Fogelman, A.M. (1992) Lipopolysaccharide-induced inhibition of scavenger receptor expression in human monocyte-macrophages is mediated through tumor necrosis factor- alpha. *J Immunol*, **148**, 112-116.

Vuorio, E. and de Crombrugghe, B. (1990) The family of collagen genes. *Annu Rev Biochem*, **59**, 837-872.

Werts, C., Tapping, R.I., Mathison, J.C., Chuang, T.H., Kravchenko, V., Saint Girons, I., Haake, D.A., Godowski, P.J., Hayashi, F., Ozinsky, A., Underhill, D.M., Kirschning, C.J., Wagner, H., Aderem, A., Tobias, P.S. and Ulevitch, R.J. (2001) Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. *Nat Immunol*, **2**, 346-352.

Whitney, G.S., Starling, G.C., Bowen, M.A., Modrell, B., Siadak, A.W. and Aruffo, A. (1995) The membrane-proximal scavenger receptor cysteine-rich domain of CD6 contains the activated leukocyte cell adhesion molecule binding site. *J Biol Chem*, **270**, 18187-18190.

Wu, H., Moulton, K., Horvai, A., Parik, S. and Glass, C.K. (1994) Combinatorial interactions between AP-1 and ets domain proteins contribute to the developmental regulation of the macrophage scavenger receptor gene. *Mol Cell Biol*, **14**, 2129-2139.

Yokota, T., Ehlin-Henriksson, B. and Hansson, G.K. (1998) Scavenger receptors mediate adhesion of activated B lymphocytes. *Exp Cell Res*, **239**, 16-22.

Yoshida, H., Hayashi, S., Kunisada, T., Ogawa, M., Nishikawa, S., Okamura, H., Sudo, T. and Shultz, L.D. (1990) The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature*, **345**, 442-444.