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Structural and functional studies of class A scavenger
receptors MARCO (SCARA2) and SCARA5

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ABSTRACT

Our bodies are constantly exposed to various microorganisms and there is a constant battle between their survival and ours. Fortunately we are equipped with our immune system that takes remarkably good care of us, but when it fails we all know the consequences. This thesis work concentrates on innate immunity and the molecular mechanisms set in place to fight infections and to keep our bodies functional. More specifically, the focus is on germline-encoded receptors MARCO and SCARA5 capable of recognizing and handling pathogen- or damage-associated molecular patterns (PAMPs and DAMPs).

Macrophage receptor with a collagenous structure (MARCO) is a pattern recognition receptor (PRR) expressed by professional phagocytes, macrophages and dendritic cells, and participates in the clearance of bacteria and pollution particles. It is a trimeric molecule with a short N-terminal cytosolic domain, a single pass transmembrane domain followed by a large extracellular region with a spacer domain, a long collagenous domain, and C-terminal scavenger receptor cysteine-rich (SRCR) domain. In this study it was established that the C-terminal SRCR domain of MARCO is the main functional unit mediating both ligand binding and adhesion.

We could show that a soluble form of MARCO binds bacteria cell wall components lipopolysaccharide and lipoteichoic acid. Utilizing the soluble protein we identified several hydrophobic peptides that bound to the SRCR domain of the receptor. The peptide sequences were identified as part of complement component C4b that functions as an opsonin once bound to the surface of bacteria. We could detect some binding of C4b to MARCO, but the peptide sequence was not involved in the binding. Another ligand, acetylated low-density lipoprotein (AcLDL), was also found to bind to the SRCR domain. With the help of mutational analysis and by solving the crystal structure of a monomeric and dimeric form of the SRCR domain to 1.78 and 1.77 Å, respectively, we could identify in more detail that a β -sheets structure with several positively charged arginines and a negative cluster residing in a long loop area of the structure were important for the ligand-binding functions. These areas affected also MARCO mediated adhesion to various surfaces. Based on the ion-binding site found in the long loop region, we were able to show that Ca^{2+} is needed for ligand binding.

Other half of the thesis work focused on the physiological function of SCARA5, a MARCO related scavenger receptor having an additional α -helical coiled coil domain between the spacer domain and the collagenous domain. Similar to MARCO, SCARA5 was found to promote cell adhesion and bind and internalize ligands such as bacteria and LPS. In contrast, we could only see limited binding of AcLDL, the knowledge of which we used in the mutational analysis made to map the ligand-binding region in the MARCO SRCR domain. Further, we utilized the strong cell adhesion for selection of stable cell lines without using antibiotics and were able to improve cell viability and handling of the cells in serum-free culture conditions during protein production. Our *in vivo* functional studies of SCARA5 revealed that in contrast to MARCO, the receptor is expressed by a vimentin and platelet-derived growth factor receptor α (PDGFR α) positive subpopulation of fibroblasts participating in immune related homeostasis. The homeostatic function was evident on the aging SCARA5 deficient mice that developed antinuclear antibodies and connective tissue related autoimmune disease-like symptoms with lymphoid cell accumulations in several organs, especially lung.

LIST OF PUBLICATIONS

- I. Chen, Y., Sankala, M., **Ojala, J.R.M.**, Sun, Y., Tuuttila, A., Isenman, D.E., Tryggvason, K., and Pikkarainen, T. A phage display screen and binding studies with acetylated low density lipoprotein provide evidence for the importance of the scavenger receptor cysteine-rich (SRCR) domain in the ligand-binding function of MARCO *J Biol Chem* 2006; 281: 12767-12775.
- II. **Ojala, J.R.**, Pikkarainen, T., Tuuttila, A., Sandalova, T., and Tryggvason, K. Crystal structure of the cysteine-rich domain of scavenger receptor MARCO reveals the presence of a basic and an acidic cluster that both contribute to ligand recognition. *J Biol Chem* 2007; 282:16654-16666.
- III. **Ojala, J.R.M.**, Pikkarainen, T., Domogatskaya, A., Tryggvason, K., and Rodin, S. A novel scavenger receptor 5-based antibiotics-independent selection method for generation of stable recombinant protein producing cell lines that is especially suitable for proteins affecting cell adhesion. *BioTechniques* 2012, 53, 221-230
- IV. **Ojala, J.R.M.**, Pikkarainen, T., Elmberger, G., and Tryggvason, K. Progressive reactive lymphoid connective tissue disease and development of autoantibodies in scavenger receptor 5 (SCARA5) deficient mice. Submitted manuscript (Accepted after revision for publication, *Am J Pathol.* May 2013)

Additional publications not included in the thesis:

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He, B., Österholm, A-M, Ojala, JR., Andersson, A-C., and Tryggvason K. A remote *cis*-acting variant at 3q links glomerular NCK1 to diabetic nephropathy. *PlosOne* 2013, In press.

TABLE OF CONTENTS

1	Introduction	1
1.1	Innate immunity.....	1
1.1.1	Antimicrobial peptides.....	1
1.1.2	Complement system.....	1
1.1.3	Effector cells.....	2
1.2	Adaptive immunity.....	3
1.2.1	T lymphocytes.....	3
1.2.2	B lymphocytes.....	4
1.2.3	Autoimmunity.....	5
1.3	Pattern recognition receptors.....	5
1.3.1	Secreted PRRs – Collectins.....	6
1.3.2	Membrane bound signaling PRRs – Toll-like receptors (TLR).....	6
1.3.3	Cytosolic signaling PRRs –Nucleotide-binding oligomerization domain (NOD)-like receptors (NLR).....	7
1.3.4	Endocytic PRRs – Mannose receptor.....	7
1.3.5	Endocytic PRRs - Class A scavenger receptors.....	8
1.3.6	The SRCR superfamily.....	12
2	Aims.....	14
2.1	Specific aims.....	14
3	Methods.....	15
4	Results.....	18
4.1	The role of MARCO SRCR domain in ligand-binding elucidated by phage display screening and Acetylated LDL binding (Paper I).....	18
4.2	Crystal structure reveals multiple binding sites in the MARCO SRCR domain (Paper II).....	21
4.3	SCARA5 expression improves viability of mammalian cells in serum-free culture conditions and can be used as an alternative to antibiotics for selecting stable protein-producing cell lines (Paper III).....	25
4.4	Deficiency of fibroblast expressed SCARA5 in mice causes autoimmune disease -like phenotype (Paper IV).....	25
5	Discussion.....	28
6	Concluding remarks.....	33
7	Acknowledgements.....	34
8	References.....	37

LIST OF ABBREVIATIONS

PAMP	Pathogen-associated molecular pattern
PRR	Pattern recognition receptor
LPS	Lipopolysaccharide
C	Complement
MBL	Mannose-binding lectin
MASP	MBL-associated serine protease
NK	Natural killer
DC	Dendritic cell
MHC	Major histocompatibility complex
CD	Cluster of differentiation
TCR	T-cell receptor
BCR	B-cell receptor
Ig	Immunoglobulin
DAMP	Danger-associated molecular pattern
SRCR	Scavenger receptor cysteine-rich
MARCO	Macrophage receptor with a collagenous structure
AcLDL	Acetylated low-density lipoprotein
PDGFR α	Platelet-derived growth factor receptor α
SP	Surfactant protein
CL	Collectin
TLR	Toll-like receptor
LRR	Leucine-rich repeat
IL	Interleukin
TIR	Toll/interleukin-1 receptor
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
LTA	Lipoteichoic acid
NOD	Nucleotide-binding oligomerization domain
NLR	NOD-like receptor
NF- κ B	Nuclear regulatory factor
FNII	Fibronectin type II
CSR	Cellular stress response

SRCL	Scavenger receptor with C-type lectin
Tesr	Testis expressed scavenger receptor
SR-A	Scavenger receptor A
Apo	Apolipoprotein
SLE	Systemic lupus erythematosus
EAE	Experimental autoimmune encephalomyelitis
UGRP1	Uteroglobin-related protein 1
CEA	Carcinoembryonic antigen
CAM	Cell adhesion molecule
DMBT1	Deleted in malignant tumors 1
GST	Glutathione S-transferase
CHO	Chinese hamster ovary
ASMA	α -smooth muscle actin
FSP	Fibroblast specific protein
DSP	Dithiobis(succinimidyl propionate)
Poly I	Polyinosinic acid
GFP	Green fluorescent protein
LN3G	Globular domain of laminin α 3 chain
CAF	Cancer-associated fibroblast

1 INTRODUCTION

Even though our bodies are constantly exposed to pathogens such as bacteria, viruses, parasites, and fungi, infections are rare and even in the case of a severe illness we usually recover within a couple of weeks. This remarkable capacity to infection resistance is accomplished by our immune system (Janeway and Medzhitov, 2002). An effective barrier against most microorganisms is provided by anatomical and physiological barriers such as the external or internal epithelial surfaces of skin, respiratory tract mucosa, gastrointestinal mucosa, and reproductive mucosa; epithelial cells are held together with tight junctions and the microorganisms are eliminated by ciliary movements, low stomach pH, and excretions such as mucus and saliva. Further protection is provided through the interplay of specialized cells and other components of innate and adaptive immunity, parts of which will be reviewed briefly in the following chapters (Turvey and Broide, 2010).

1.1 INNATE IMMUNITY

Innate immunity is an evolutionarily ancient way of defending the organism and many of the components involved are present from mammals to plants and simple multicellular organisms (Hoffmann et al., 1999; Janeway and Medzhitov, 2002; Uthaisangsook et al., 2002). Innate immunity relies on the recognition of distinct molecular motifs, pathogen-associated molecular patterns (PAMP), such as microbial proteins, lipids, nucleic acids, and carbohydrates. The recognition is mediated mainly by complement, antimicrobial peptides, and specialized pattern recognition receptors (PRR) on effector cells (Bardoel and Strijp, 2011; Olive, 2012). Other soluble mediators (humoral components) such as C-reactive protein, lipopolysaccharide (LPS) binding protein, and collectins are also involved (Turvey and Broide, 2010).

1.1.1 Antimicrobial peptides

Antimicrobial peptides found throughout the eukaryotic kingdom function by direct recognition of microbial structures and next to instant killing of the bacteria, but require high local concentrations for efficient action (Bardoel and Strijp, 2011; Hoffmann et al., 1999). They vary in length from 5-60 amino acid residues, but are mostly cationic in nature. Most antimicrobial peptides adopt an antiparallel β -sheet structure with a possible α -helix held together by multiple disulfide bonds (Brogden, 2005; Hoffmann et al., 1999).

1.1.2 Complement system

Complement (C) system is composed of more than 40 soluble and membrane bound proteins that participate in the elimination of microorganisms, immune complexes, and apoptotic cells. Furthermore, they enhance and direct adaptive immunity through opsonization and leukocyte chemotaxis (Ricklin et al., 2010). There are three alternative pathways to activate the complement system but they all converge at one central step: the cleavage of complement component C3 (Figure 1). After C3 cleavage, the cascade continues and leads to the formation of a C5b-9 membrane attack complex

capable of lysing the target cell (Bardoel and Strijp, 2011). Recently it has been shown that even some proteases such as plasmin and thrombin can activate C3. Furthermore, under some conditions mannose-binding lectin (MBL) can directly activate C3 (Ricklin et al., 2010).

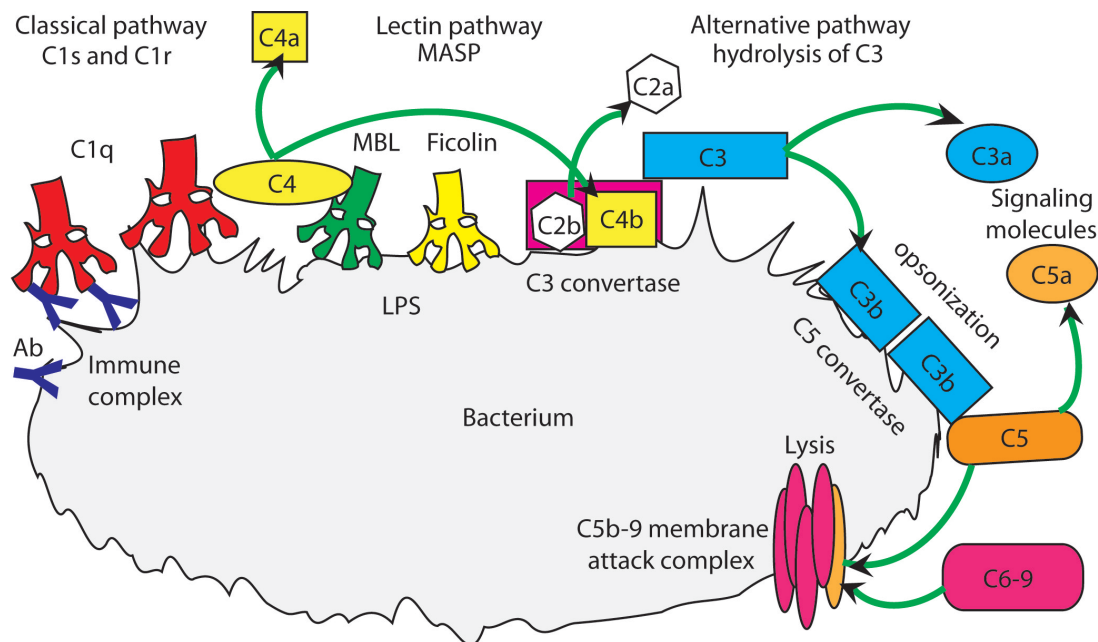


Figure 1. Overview of the complement system. Complement is activated by either the classical, lectin, or alternative pathway. Classical pathway is activated after C1q (red) recognizes the bacterium either directly or through an antibody (dark blue) immune complex. The recognition leads to the cleavage of C4 into C4a and C4b (yellow) by a C1 complex (C1q, C1r, and C1s). C4b is attached to the surface (opsonization) and binds C2, which in turn leads to generation of C2a, C2b (white) and C3 convertase (C4b2b, purple) capable of cleaving C3 (light blue). In the alternative pathway the hydrolysis of C3 can occur spontaneously. The mannose-binding lectin (MBL) pathway is activated by recognition of carbohydrate-containing structures (e.g. LPS) via MBLs (green) or ficolins (yellow), which are bound to MBL-associated serine proteases (MASPs) capable of creating the C3 convertase needed to cleave C3 (Ricklin et al., 2010). The activation of C3 leads to the formation of a signaling molecule C3a and C3b, which is deposited on the target surface (opsonization). Opsonization marks the bacterium for phagocytosis, but the complement cascade continues by cleavage of C5. C5a attracts phagocytes while C5b is the first component of the membrane attack complex (C5b-9) capable of lysing bacteria (Bardoel and Strijp, 2011).

1.1.3 Effector cells

The main effector cells in innate immunity are phagocytes (macrophages, monocytes, dendritic cells, and neutrophils), which can kill the bacteria within minutes. However other hematopoietic cells such as mast cells, eosinophils, and natural killer (NK) cells are involved. Further assistance is provided by fibroblasts, endothelial cells, and epithelial cells (Turvey and Broide, 2010).

Neutrophils are the most abundant (~60 % of all the leucocytes) and the most important phagocytes normally circulating in blood. Any hereditary defects in their function lead to life threatening infections. Upon infection, they rapidly migrate to the site and kill the bacteria by releasing a combination of proteases, lipases, antimicrobial peptides, amidases, and highly reactive oxygen species (Bardoel and Strijp, 2011).

Macrophages and dendritic cells (DC) are both derived from circulating monocytes and reside in most tissues surveying the environment for any foreign or

harmful antigens. Upon contact with macrophage receptors, antigens are ingested through phagocytosis and processed for antigen presentation on major histocompatibility complex (MHC) I and II molecules. After encountering the antigens (exogenous or endogenous), DCs rapidly migrate to local lymphoid tissue where the antigens are cross-presented to passing immature T4- and T8-lymphocytes causing them to differentiate. In contrast, macrophages are either recruited to the infection site or they exert their function locally at different sites i.e. peritoneal macrophages, alveolar macrophages, Kupffer cells of the liver, marginal zone macrophages of the spleen, and others. Macrophages present the antigens to effector T4-lymphocytes, which have already encountered the antigens before (DC contact), and start producing cytokines and chemokines to support the inflammatory response (Janeway et al., 2001).

Most other hematopoietic cell types involved in innate immune responses have more immunomodulatory roles and they excrete different substances e.g. antimicrobial peptides or histamine from mast cells that can either kill the bacteria or work as chemokines or cytokines (Janeway et al., 2001). NK cells are a specialized cell type circulating in the body. They constantly survey the expression level of MHC-I molecules normally expressed on all other cell types. If a cell shows abnormal expression of MHC-I molecules, NK cell can induce a direct destruction of the cell and elimination of the threat (Stanietsky and Mandelboim, 2010).

The nonhematopoietic cells function also as immunomodulators. Most epithelial layers are covered by mucus containing a repertoire of antimicrobial agents. On the other hand, endothelial cells express upon induction a repertoire of membrane bound receptors (selectins, vascular cell adhesion molecule-1) facilitating the migration of other immune cells to the infection site (Szmítko et al., 2003). All these cell types, including fibroblasts, express MHC-I molecules and cluster of differentiation (CD)40, which is an important costimulatory molecule needed for the interaction with effector T4-lymphocytes (Vogel et al., 2004). Furthermore, a circulating fibroblasts population, fibrocytes, capable of antigen presentation has been identified (Bucala et al., 1994; Chesney et al., 1997). In addition, fibroblasts act as support cells and hold together the integrity of lymphoid organs and thereby support the differentiation of various leukocytes (Roozendaal and Mebius, 2011).

1.2 ADAPTIVE IMMUNITY

Adaptive immunity relies on B and T lymphocytes and as briefly mentioned earlier receive help from the molecules and cells involved in innate immunity. The strength of the system is the ability to generate specific receptors and antibodies that have the capacity to recognize virtually any antigen. Because of the specificity, the process eliminating the antigens takes much longer (up to 5 days) than the immune reactions described earlier as part of the innate immunity (minutes, hours). However, adaptive immunity has memory and antigens encountered for the second time are eliminated rapidly. As a drawback, there is a risk of developing receptors and antibodies recognizing self-proteins and autoimmunity (Turvey and Broide, 2010).

1.2.1 T lymphocytes

T lymphocytes are assisting adaptive immunity through cell-mediated responses. There are two main types of T cells, which differ in their mode of action. The cell populations

expressing a surface molecule CD4 are considered as helper T cells and upon activation assist macrophages to kill bacteria, support the production of antibodies, regulate tolerance against self-proteins, and confer memory. The activated CD8 positive T cells, also known as cytotoxic T cells, kill affected cells by inducing apoptosis (Janeway et al., 2001).

1.2.1.1 T cell development and specificity

Precursors of T lymphocytes originate from bone marrow and migrate into the thymus, where they first mature either into CD4 or CD8 positive cells through a positive selection process. In this selection process, only cells expressing T-cell receptor (TCR) proteins capable of recognizing MHC molecules survive. The cells recognizing MHC-I and MHC-II molecules become CD8 and CD4 positive, respectively. TCR receptor specificity is established by random gene segment rearrangements and is the key behind the recognition of virtually any ligand (Janeway et al., 2001; Sebzda et al., 1999).

At this stage the cells are still able to recognize both foreign and self-antigens, and therefore they undergo a negative selection process. During this process the cells are challenged with a variety of self-antigens. In order to avoid autoimmune reactions, the self-reacting cells are eliminated (Janeway et al., 2001; Sebzda et al., 1999).

Once T cells have passed the selection processes, they exit the thymus as naïve T cells and start circulating in the blood. Once the naïve T cells encounter DCs presenting the antigen recognized by the TCR, they become activated, start dividing, and become effector T cells, which again circulate in the blood and assist macrophages and B cells presenting the same antigen (CD4 positive cells, MHC-II recognition) or kill the affected cells (CD8 positive cells, MHC-I recognition) (Janeway et al., 2001).

1.2.2 B lymphocytes

B lymphocytes are behind the humoral response of adaptive immunity through their ability to produce antibodies against virtually any antigen. Unlike T lymphocytes, B cells are also antigen presenting cells (MHC-II) and require assistance from helper T cells (CD4 positive) in order to become antibody-producing plasma cells (Janeway et al., 2001).

1.2.2.1 B cell development and specificity

Just like T lymphocytes, B lymphocytes originate in the bone marrow. Similar to T cell development, they go through a positive and negative selection process, but instead of migrating to the thymus they stay in the bone marrow. During the positive selection process B cell precursors go through gene fragment rearrangements resembling those happening to TCRs. This process ensures that the maturing B cells will express a vast number of functional B-cell receptors (BCR) on their surfaces. BCRs are membrane bound immunoglobulins (Ig), which determine the specificity of the plasma cell secreted antibodies (Ig). During the negative selection process, the newly formed immature B cells recognizing self-antigens are eliminated (Janeway et al., 2001; Monroe et al., 2003).

The immature B cells expressing BCR exit the bone marrow and start circulating in the blood. When they are presented with BCR specific antigens in secondary lymphoid organs, they become activated and start presenting the antigens on their MHC molecules. A second activation step is required from a CD4 positive effector T cell before the cell can become antibody producing plasma cell. Further, this interaction enables the B cells to produce different classes of immunoglobulins and undergo additional fine-tuning of the expressed antibody sequences in order to improve the affinity between antibody-antigen complexes (Janeway et al., 2001).

1.2.3 Autoimmunity

Autoimmune disorders are characterized by conditions where the immune system is activated to react against self-antigens. As indicated above, this process is usually regulated tightly by negative selection of self-reactive B and T cells during their development. However, not all the self-antigens are present in the environment of “schooling” and some self-reactive cells escape into the blood stream. Fortunately the effects of many of these cells are suppressed by the action of regulatory T cells (Tregs) (Tian et al., 2012). Additional protection is achieved by compartmentalization; many of these cells are reactive against intracellular or nuclear macromolecules and will therefore never become activated (Kamradt and Mitchison, 2001). Even though defects in the regulation of adaptive immunity play a major role in the development and maintenance of autoimmune diseases, innate immune responses contribute to the disease by different mechanisms. For example there is compelling evidence that excessive activation of complement leads to many different types of autoimmune diseases (Meri, 2007).

Currently there are over 70 known chronic autoimmune/autoinflammatory diseases. Through genetic association studies some of the susceptibility genes have been identified, but in more than 60 % of the patients the cause is unknown. Identification of the gene defects is difficult, because many of the diseases are caused by multiple gene mutations. Furthermore, environmental factors play a major role in the disease development. This is evident, since in many cases only one of identical twins develops the disease, even though they share the genetic code (Doria et al., 2012).

1.3 PATTERN RECOGNITION RECEPTORS

As indicated above, the effector cells of innate immunity express a limited number of germline encoded pattern recognition receptors (PRRs) that recognize conserved structures on the pathogens (PAMPs) or danger-associated molecular patterns (DAMPs) including endogenous molecules released from e.g. dying cells after tissue damage (Olive, 2012). However, the concept of pattern recognition includes even soluble proteins such as MBL, other complement proteins (C3b, C4b), C-reactive protein, lung surfactant proteins (SP-A and SP-D), and other collectins. Furthermore, some of these receptors are found even on B and T cells (Janeway and Medzhitov, 2002; Turvey and Broide, 2010). Finally, apart from recognizing the patterns, some of the PRRs are able to mediate the inflammatory responses by inducing signaling pathways leading to the production of cytokines and chemokines (Iida et al., 2001). Next, some of the different types of PRRs will be reviewed with special emphasis on class A scavenger receptors.

1.3.1 Secreted PRRs – Collectins

Collectins are a group of PRRs consisting of an N-terminal cysteine-rich domain, a collagenous domain, an α -helical coiled-coil neck domain and a C-terminal C-type lectin-like domain. They form trimers through their collagenous and coiled-coil regions and further oligomerize through the N-terminal cysteine-rich domain into tetrameric or hexameric multimers illustrated by rotary shadow imaging (Hansen and Holmskov, 1998). The group of soluble collectins in humans includes liver and lung derived MBL, SP-A, SP-D as well as liver and kidney collectins (CL-L1 and CL-K1, respectively). In addition, a number of other collectins have been identified in other organisms e.g. amphioxus expresses 66 different collectins. (Ohtani et al., 2012).

Similar to the MBL function described as part of the complement system, all the members of collectins play a part in the innate immune system. They recognize carbohydrate structures on microorganisms in a Ca^{2+} -dependent manner via their C-type lectin-like domains. Once bound to the microorganisms, they are recognized and engulfed by opsonin receptors such as complement receptor 3 (CD11b/CD18) expressed on the phagocytes. There are several factors contributing to the number of microorganisms recognized by collectins. First, specificity and affinity toward different carbohydrates vary between the collectins (Kishore et al., 2006; Ohtani et al., 2012). Second, specificity is affected by spacing of the lectin domains in the oligomerized proteins; SP-D is 46 nm long while SP-A is only 20 nm long (Hansen and Holmskov, 1998). Third, the oligomerization increases binding affinity; a single lectin domain can be eluted with 0.1 mM glucose in contrast to 20 mM glucose needed for the oligomerized form (Hoppe and Reid, 1994).

1.3.2 Membrane bound signaling PRRs – Toll-like receptors (TLR)

TLRs, named after the homologous protein Toll found in *Drosophila melanogaster*, are type I membrane proteins responsible for translating the recognition of PAMPs or DAMPs into signals leading to the activation of immune system (Lemaitre et al., 1996). They interact with many other PRRs and mediate signaling between innate and adaptive immunity. TLRs are widely expressed in epithelial and endothelial cells as well as in macrophages, neutrophils, and dendritic cells. To date, thirteen mammalian TLRs have been identified, but only ten homologues are expressed in humans. As membrane proteins, they can be found either on the plasma membrane (TLRs 1, 2, 4, 5, 6 and 11) or intracellular compartments (TLRs 3, 7, 8 and 9) (Kawai and Akira, 2011; West et al., 2006). They possess an extracellular ectodomain composed of 19 to 25 leucine-rich repeats (LRR) responsible for the ligand-binding activity, a single transmembrane helix, and an intracellular signaling domain known as Toll/interleukin (IL)-1 receptor (TIR) domain (Pandey and Agrawal, 2006). Structural studies revealed that the ligand-binding LRR ectodomain adopts a horseshoe-like shape capable of dimerization. Indeed, the receptors functions either as homo- or heterodimers (Kang and Lee, 2011).

Different TLRs are able to bind directly or indirectly to a vast number of DAMPs or PAMPs derived from viruses, bacteria, mycobacteria, fungi, or parasites. Briefly, different PAMPs include lipoproteins, double- and single-stranded ribonucleic acid (RNA), flagellin, LPS, and deoxyribonucleic acid (DNA) (Kawai and Akira, 2011).

More specifically, TLR1/2 heterodimers are able to bind triacyl lipopeptides while TLR2/TLR6 heterodimers recognize diacylated bacterial lipoproteins, lipoteichoic acid (LTA), and fungi. Homodimeric TLR2 binds to various bacterial, fungal or parasitic peptidoglycans and viral protein hemagglutinin. The intracellular TLR3 is the main receptor for viral double-stranded RNA while single-stranded RNA is the ligand for TLR7 and TLR8. TLR4 is the main receptor for LPS but requires the presence of an adaptor protein MD2 and CD14 for signal transduction. The ligand for TLR5 is flagellin. TLR9, on the other hand, is the receptor for bacterial DNA. TLR11 binds to parasitic profilin and an unknown ligand on uropathogenic bacteria (Olive, 2012; West et al., 2006).

After recognizing their cognate PAMPs, the TLR receptors dimerize and induce microbe specific signaling pathways through their intracellular TIR domains. The specificity depends on the recruitment of different adapter proteins with another TIR domain. There are five known adapters: myeloid differentiation factor primary-response gene 88 (MyD88), TIR-domain-containing adapter inducing interferon β (TRIF), TIR-domain-containing adapter protein (TIRAP), TRIF-related adapter molecule (TRAM), and sterile- α and Armadillo motif containing protein (SARM). Through complex interactions, the signaling pathways culminate in the activation of NF- κ B (nuclear regulatory factor) or interferon regulatory factors (IRFs), which in turn induce the production of different cytokines and chemokines (Kawai and Akira, 2011; Olive, 2012).

1.3.3 Cytosolic signaling PRRs –Nucleotide-binding oligomerization domain (NOD)-like receptors (NLR)

The NLR family of proteins are cytosolic signaling molecules recognizing a wide array of bacterial ligands and DAMPs mainly associated with cell death. They were first discovered when NF- κ B pathway induction was seen after infection with some invasive bacteria e.g. *Shigella flexneri* (Fritz et al., 2006). Currently there are 23 known NLRs in humans. All these proteins consist of C-terminal LRR domain implicated in ligand binding, a central nucleotide-binding site (NBS or NACHT), and an N-terminal protein-protein interaction domain composed of either CARD (caspase-activating and recruitment domain), PYD (pyrin domain) or BIR (baculovirus inhibitor of apoptosis protein repeat). Nod1 and Nod2 were the first identified NLR family members. They both recognize subunits of bacterial peptidoglycan and induce either the NF- κ B signaling pathway or mitogen-activated protein kinases (MAPKs). Upon ligand binding, most other NLRs lead to the activation of caspase-1-activating inflammasomes. Caspase-1 signaling pathway leads either to the production of IL-1 β and IL-18 or to cell death (Fritz et al., 2006; Olive, 2012).

1.3.4 Endocytic PRRs – Mannose receptor

Mannose receptor belongs to a C-type lectin-like receptor superfamily. Most of these receptors bind to carbohydrates in a Ca²⁺-dependent manner, but some have lost the ability to bind Ca²⁺ and bind non-carbohydrate ligands. Structurally these proteins vary from receptors with a single or multiple copies of lectin-like domains to membrane bound and soluble collectins described earlier (Zelensky and Gready, 2005). Mannose receptor is type I transmembrane receptor composed of N-terminal cysteine-rich

domain followed by a single fibronectin type II domain (FNII), eight copies of C-type lectin-like domains, a transmembrane domain, and a short C-terminal cytoplasmic domain. It is expressed by most differentiated macrophages, lymphatic and sinusoidal endothelium, mesangial kidney cells, tracheal smooth muscle cells, and the epithelium of the retinal pigment (Pluddemann et al., 2006).

As indicated by its name, mannose receptor binds several ligands bearing terminal mannose residues. More specifically, the ligands are terminal L-fucose, D-mannose, D-N-acetylglucosamine, and D-galactose. Typical targets are various microorganisms, such as *Candida albicans*, *Pneumocystis carinii*, *Leishmania donovani*, *Mycobacterium tuberculosis*, and *Klebsiella pneumoniae*. Mammalian glycoproteins seldom have terminal mannose or N-acetylglucosamine residues commonly found in the surface of microorganisms explaining the recognition between self and non-self and a role in immunity (East and Isacke, 2002).

1.3.5 Endocytic PRRs - Class A scavenger receptors

Scavenger receptors are membrane bound PRRs originally defined by their ability to bind and endocytose modified low-density lipoproteins (LDL), such as oxidized and acetylated LDLs (OxLDL and AcLDL) (Brown and Goldstein, 1983). Initial studies concentrated on their role in atherosclerosis, but lately they have been recognized as multiligand receptors for a variety of artificial or natural polyanionic ligands as well as many microorganisms and apoptotic cells. Scavenger receptors are a diverse group of proteins classified in groups (A-H) based on their multidomain structures, but here only the Class A scavenger receptors will be reviewed. (Areschoug and Gordon, 2008). The general domain organisation of these receptors is shown in Figure 2. Briefly, they are all trimeric type II membrane proteins with a short N-terminal intracellular domain, a single pass transmembrane domain, a short spacer region followed by varying lengths of α -helical coiled-coil domains and/or collagenous domains, and an optional C-terminal scavenger receptor cysteine-rich (SRCR) domain or a C-type lectin domain.

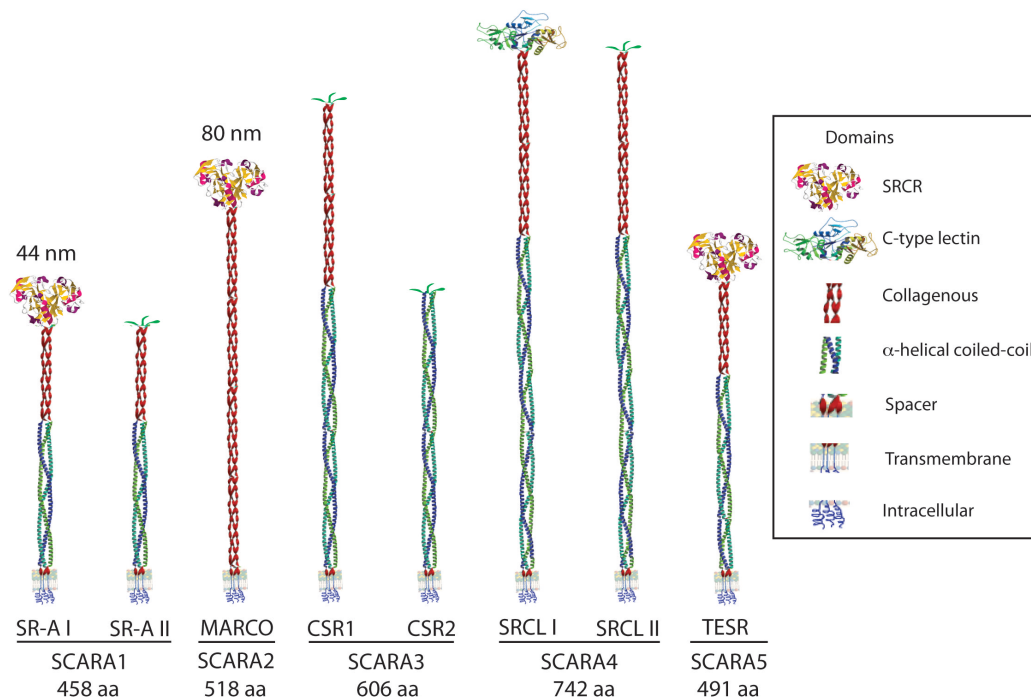


Figure 2. Domain structure of class A scavenger receptors. There are five members of class A scavenger receptors (SCARA1-5): scavenger receptor A (SR-A I, 454 amino acids (aa)), macrophage receptor with a collagenous structure (MARCO, 518 aa), cellular stress response (CSR, 606 aa), scavenger receptor with C-type lectin (SRCL I, 742 aa) and testis expressed scavenger receptor (Tcsr, 491 aa) (Elomaa et al., 1995; Han et al., 1998; Kodama et al., 1990; Nakamura et al., 2001; Sarraj et al., 2005). Three of the receptors have splice variants (SR-A II, CSR2 and SRCL II). The receptors are composed of a short intracellular domain, a transmembrane domain, a spacer domain and various lengths of α -helical coiled-coil domains and collagenous domains. The C-terminus may or may not include an SRCR domain or a C-type lectin domain. The lengths of all the receptors depicted in the figure are based on the measurements made with negative staining and rotary shadowing microscopy for SR-A I (44 nm) and MARCO (80 nm) (Resnick et al., 1996; Sankala et al., 2002).

1.3.5.1 Scavenger receptor A (SR-A, SCARA1)

SR-A was the first member of class A scavenger receptors to be cloned. It is also the founding member of the SRCR domain superfamily of proteins (Freeman et al., 1990; Kodama et al., 1990). It is a trimeric 220-250 kDa protein constitutively expressed in most tissue macrophages, DCs, and certain endothelial cells (Peiser et al., 2002). It exists as two functional splice variants I and II, and an additional isoform III trapped within the endoplasmic reticulum that may have a dominant-negative regulatory function (Gough et al., 1998). As indicated in Figure 2, SR-A II lacks the C-terminal SRCR domain. Similarly, the III splice variant lacks the first half of the SRCR domain making it unable to fold properly. Despite the structural difference, both SR-A I and SR-A II have similar ligand-binding properties; binding studies with transfected cells have shown differential binding affinity, but the expression levels might have been different (Peiser et al., 2000; Peiser et al., 2006). However, a recent report indicates that the SRCR domain is a binding site for complement component iC3b (Goh et al., 2010). Otherwise, the ligand-binding region has been localized to a positively charged lysine-rich region at the C-terminus of the collagenous domain. Positive charge is required, since most of the ligands identified thus far are polyanionic in nature (Doi et al., 1993). Electron microscopy and rotary shadowing of soluble SR-A I and SR-A II molecules suggest that the protein is around 44 nm long but it is flexible and can bend with angles ranging from 0° to 180° between the α -helical coiled-coil domain and the collagenous domain (Resnick et al., 1996).

The first identified ligands for SR-A were modified LDL molecules often found in atherosclerotic plaques. Since then the role of SR-A in the development of atherosclerosis has been extensively studied. Indeed, SR-A expression was found in the foam cells of the plaques, and the sizes of the atherosclerotic lesions were smaller in apolipoprotein E (ApoE)/SR-A or LDL receptor/SR-A double deficient mice than in ApoE or LDLR single knockouts (Babaev et al., 2000; Sakaguchi et al., 1998; Suzuki et al., 1997). However, somewhat contradictory results have been reported since then (Makinen et al., 2010; Manning-Tobin et al., 2009; Moore et al., 2005). Nevertheless, other evidence suggests a role for SR-A in lipid metabolism; ApoA-I and ApoE were found to be ligands for SR-A and *in vivo* overexpression of SR-A affects the serum lipid levels (Gao et al., 2003; Neyen et al., 2009).

The first indication that SR-A has a role in the host defense came when it was found to bind the lipid A moiety of LPS (Hampton et al., 1991). Since then it has been shown to bind LTA, bacterial CpG DNA, double-stranded RNA, and live Gram-negative and Gram-positive bacteria such as *Escherichia coli*, *Staphylococcus aureus*

and *Neisseria meningitides* (Greenberg et al., 1996; Limmon et al., 2008; Peiser et al., 2000; Peiser et al., 2002; Zhu et al., 2001). Studies made with SR-A deficient mice have demonstrated that SR-A confers protection against viral and bacterial infections such as herpes simplex virus 1 and Gram-positive bacteria *Listeria monocytogenes*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* as well as Gram-negative bacteria *Neisseria meningitides* (Arredouani et al., 2006; Pluddemann et al., 2009; Suzuki et al., 1997; Thomas et al., 2000). In contrast, a recent study shows that SR-A deficient mice are more resistant to polymicrobial induced sepsis (Ozment et al., 2012). The discrepancy can maybe be explained by the differences in the experimental models and how they induce different signaling pathways; SR-A has been shown to interact with cell surface TLR4 and endosomal TLR3 and TLR9 as well as intracellular NLR receptors in a competitive manner, and depending on the ligand pattern different signaling pathways are activated (Mukhopadhyay et al., 2011; Ohnishi et al., 2011; Ozment et al., 2012; Yew et al., 2010).

Additional studies have shown that SR-A may play a role in the development of Alzheimer's disease, diabetes, systemic lupus erythematosus (SLE), and viral hepatitis suggesting a role in inducing different autoimmune disorders (Horiuchi et al., 2005). Indeed, SR-A deficient macrophages showed reduced uptake of apoptotic cells, SR-A is regulating antigen transfer from B cells to DCs, and SR-A deficient mice show differential progression of experimental autoimmune encephalomyelitis (EAE) (Levy-Barazany and Frenkel, 2012; Platt et al., 1996; Raycroft et al., 2012).

1.3.5.2 Macrophage receptor with a collagenous structure (MARCO, SCARA2)

MARCO was the second class A scavenger receptor to be identified. Normally, its expression is restricted to the marginal zone macrophages of the spleen, macrophages of the medullary cord of lymph nodes, and peritoneal macrophages (Elomaa et al., 1995). Strikingly, its expression is upregulated in most tissue macrophages upon bacterial infections (Elomaa et al., 1998; van der Laan et al., 1997). This induction seems to be dependent on TLRs (Chen et al., 2010; Doyle et al., 2004; Mukhopadhyay et al., 2004). Similar to SR-A, MARCO is a 210 kDa trimeric protein, but adopts a rigid, 80 nm long rod-like structure as evidenced by negative staining and rotary shadowing microscopy. Interestingly, some of the molecules oligomerize similar to what is seen for collectins (Hansen and Holmskov, 1998; Sankala et al., 2002). In contrast to SR-A, the ligand-binding domain in MARCO resides in the C-terminal SRCR domain. First, antibodies directed against the domain could block ligand binding (van der Laan et al., 1999). Second, deletion mutants of the human and mouse MARCO without the SRCR domain were unable to bind ligands (Brannstrom et al., 2002; Elomaa et al., 1998). Third, the mutant proteins without the domain were unable to induce the formation of lamellipodia-like structures and long dendritic processes typical for MARCO transfected cells (Pikkarainen et al., 1999).

Induction of MARCO expression in most tissue macrophages after an infection suggests a role for this receptor in host defense. Indeed, MARCO was shown to bind *E.coli* and *S. aureus*, but not yeast (zymosan) (Elomaa et al., 1995). Furthermore, capturing of heat killed bacteria by marginal zone macrophages could be inhibited with antibodies directed against the SRCR domain (van der Laan et al., 1999). Other bacterial ligands include LPS and CpG DNA (Jozefowski et al., 2006; Sankala et al., 2002). Functional *in vivo* studies have shown that MARCO deficient mice are more

susceptible to *Streptococcus pneumoniae* and *Clostridium sordellii* infections (Arredouani et al., 2004; Thelen et al., 2010). Furthermore, MARCO expression gives protection against *Leishmania major* infection in CBA/J mice derived macrophages (Gomes et al., 2009). Paradoxically, MARCO deficient mice are more resistant to endotoxin (LPS) induced septic shock and influenza A virus pneumonia (Chen et al., 2010; Ghosh et al., 2011). This may be explained by the differential induction of TLR4, TLR3 and NLR mediated signal transduction (Mukhopadhyay et al., 2011).

In addition to typical host defense functions, alveolar macrophages expressing MARCO participate in other scavenging functions such as binding of unopsonized pollution particles, oxidized lipids, and uteroglobin-related protein 1 (UGRP1) secreted by Clara cells (Arredouani et al., 2005; Bin et al., 2003; Dahl et al., 2007; Palecanda et al., 1999). MARCO deficient mice are also more sensitive to ovalbumin/aerosol challenge mimicking allergic asthma (Arredouani et al., 2007). Further, in contrast to its human variant mouse MARCO shows avid binding to AcLDL (Elomaa et al., 1995; Elshourbagy et al., 2000).

MARCO seems to play a role also in the development of some autoimmune diseases. It was shown that synovial fluid mononuclear cells from rheumatoid arthritis patients express high levels of MARCO (Seta et al., 2001). In addition, SLE patients generate autoreactive antibodies against MARCO leading to impaired apoptotic cell clearance, (Chen et al., 2011; McGaha et al., 2011; Rogers et al., 2009; Wermeling et al., 2007).

1.3.5.3 Cellular stress response (CSR, SCARA3)

CSR was cloned after its expression was shown to be upregulated in normal human fibroblasts in response to oxidative stress (UV irradiation or hydrogen peroxide). Pretreatment with antioxidants prevented the induction (Han et al., 1998). The receptor was localized to the endoplasmic reticulum, but a recent uptake study with negatively charged peptide complexes suggests, but does not confirm, that some of the receptors might come to the cell surface of HeLa cells (Ezzat et al., 2012). It was also shown that C-terminal residues 440-543 of CSR1 interact with cleavage and polyadenylation-specific factor 3 (CPSF3), which converts heteronuclear RNA to mRNA. The interaction induces translocation of the complex from cell nucleus to cytosol and blocks the polyadenylation process. In this way, CSR is able to suppress tumor growth and induce apoptosis (Zhu et al., 2009). CSR has also been shown to have a role in ovarian cancer, reduce the growth of tumor cells *in vitro* and in xenograft tumors as well as inhibit the growth of prostate cancer cells by inducing apoptosis through interaction with X-linked inhibitor of apoptosis protein (XIAP); C-terminal residues 513-572 of the collagenous region are needed for the interaction (Bock et al., 2012; Yu et al., 2006; Zheng et al., 2012).

1.3.5.4 Scavenger receptor with C-type lectin (SRCL, SCARA4)

SRCL is also known as CL-P1, a collectin isolated from placenta (Ohtani et al., 2001). It could also be regarded as part of the C-type lectin superfamily, because of its C-terminal lectin domain, but apart from that, the structure resembles that of the other class A scavenger receptors (Figure 2). The receptor is widely expressed by vascular endothelial cells. The human protein was also detected in alveolar macrophages and

trophoblasts (Nakamura et al., 2001; Ohtani et al., 2001; Selman et al., 2008). It is capable of binding and internalizing bacteria (*Escherichia coli*, *Staphylococcus aureus*), yeast (*Saccharomyces cerevisiae*), fungi as well as oxidized LDL, but not AcLDL. The uptake can be blocked with cytochalasin D, wortmannin, poly I, and dextran sulfate (Jang et al., 2009; Nakamura et al., 2001; Ohtani et al., 2001).

Many of the ligands bind to the collagenous sequence, but the receptor contains also the C-type lectin domain that binds multiple ligands containing lewis(x) trisaccharides (Gal β 1-4(Fuca1-3)GlcNAc). These ligands include leukocyte and tumor cell expressed proteins such as carcinoembryonic antigen (CEA), CEA-related cell adhesion molecule 1 (CEACAM1), carcinoma associated T antigen and Tn antigen as well as lactoferrin and matrix metalloproteinase-8 and -9 released from secondary granules of neutrophils (Coombs et al., 2005; Feinberg et al., 2007; Graham et al., 2011; Samsen et al., 2010; Yoshida et al., 2003).

1.3.5.5 Testis expressed scavenger receptor (*Tesr*, *SCARA5*)

SCARA5 was first described as a highly expressed protein in the testis, hence the name *Tesr*. With *in situ* hybridization studies on embryonal samples additional expression was detected in the ovary, brain, eye, head, heart, neural arch, and cartilage primordium (Sarraj et al., 2005). Later real time PCR and *in situ* hybridization studies on adult mouse tissues revealed additional expression of SCARA5 in the epithelium of bladder, lung, trachea, and small intestine (Jiang et al., 2006). In contrast, we (paper IV) and others have shown that SCARA5 is mainly expressed by mesenchymal cells, more specifically a subpopulation of fibroblasts (DeWitte-Orr et al., 2010; Li et al., 2009).

Regardless of the expression pattern, SCARA5 functions as a scavenger receptor and readily binds heat killed *E.coli* and *S. aureus*, but not zymosan or modified LDL (Jiang et al., 2006). SCARA5 is also able to bind and endocytose ferritin and haptoglobin-hemoglobin complexes suggesting a role in iron metabolism (Li et al., 2009). Interestingly, in a genome-wide association study SCARA5 expression was associated with increased levels of coagulation Factor VIII in plasma and a patient with mild iron overload was found to have a R471H mutation in SCARA5 (Antoni et al., 2011; Lee et al., 2012). In addition, two siRNA studies indicate, but do not confirm, that SCARA5 binds double-stranded viral RNA and negatively charged cell penetrating peptide complexes (DeWitte-Orr et al., 2010; Ezzat et al., 2012). It has also been suggested that *SCARA5* acts as a tumor suppressor gene. The expression of SCARA5 was downregulated in several tumor samples and tumor cell lines after promoter hypermethylation. Both *in vitro* and *in vivo* studies suggest that SCARA5 expression suppresses tumor cell growth, invasiveness, and reduces the number of metastases. The opposite was seen with reduced SCARA5 expression (Huang et al., 2010; Khamas et al., 2012; Yan et al., 2012).

1.3.6 The SRCR superfamily

SRCR domain is an ancient and highly conserved domain of ~110 residues first identified in SR-A (Freeman et al., 1990). Since then, the domain has been found in numerous either soluble or membrane bound proteins often associated with the innate immune system. Additional variation to the SRCR superfamily brings the fact that many of the proteins are multimeric and may contain single copies or tandem repeats of

the domain (Martinez et al., 2011; Resnick et al., 1996). The immunological role of this domain seems evident, since the purple sea urchin, *Strongylocentrotus purpuratus*, which lacks the adaptive immune system, contains 1,095 tentative SRCR domain copies distributed to 218 genes (Rast et al., 2006).

Depending on the number and position of cysteine residues (6-8), as well as based on the exon-intron organization, the SRCR domains have been divided into two subclasses (A and B) (Freeman et al., 1990; Resnick et al., 1996; Sarrias et al., 2004). Structurally both of these subclasses adopt a similar globular fold with a curved β -sheet cradling a single α -helix (Garza-Garcia et al., 2008; Hohenester et al., 1999; Rodamilans et al., 2007).

Despite the large number of proteins with SRCR domains, there are only a few examples where function has been assigned to this domain. As mentioned earlier, the SRCR domain of MARCO participates in ligand binding and is involved in the induction of morphological changes in the cells (Brannstrom et al., 2002; Elomaa et al., 1998; Pikkarainen et al., 1999; van der Laan et al., 1999). Similarly, the SRCR domain of SR-AI was found to bind iC3b (Goh et al., 2010). Another example is the SRCR3 domain of human lymphocyte cell surface receptor CD6 that interacts with the activated leukocyte-cell adhesion molecule (ALCAM/CD166) (Patel et al., 1995). Also, gp-340/DMBT1 (deleted in malignant tumors 1) that binds and agglutinates *Streptococcus mutans* and various other bacterial strains is able to do so through a peptide sequence QGRVEVLYRGSWGTVVC included in eight of its 14 SRCR domains (Bikker et al., 2004; Bikker et al., 2002). The SRCR domains of DMBT1 contain even a second peptide sequence DDSWDTNDANVVCRQLGA able to bind hydroxyapatite (Bikker et al., 2013). Furthermore, the first SRCR domain in SP α , a soluble human glycoprotein expressed by several types of macrophages, binds to *Escherichia coli* and *Staphylococcus aureus* (Sarrias et al., 2005).

2 AIMS

The overall aim of the investigations presented in this thesis was to study the structural and functional properties of class A scavenger receptors MARCO and SCARA5. In the first half of the thesis the aim was to study how MARCO and its functional SRCR domain is able to bind various ligands such as bacterial cell wall components LPS and LTA. The aim of the second half of the thesis was to get insight into the function of previously uncharacterized, structurally related protein SCARA5.

2.1 SPECIFIC AIMS

The specific aims of this thesis project were to:

1. Identify potential new ligands for MARCO using a phage display screen and to study the biochemical ligand-binding properties of MARCO with BIAcore. Furthermore, we wished to narrow down the ligand-binding region in the SRCR domain of MARCO.
2. Gain insight into which amino acid residues are responsible for ligand binding in the SRCR domain of MARCO by determining the crystal structure of the domain.
3. To explore the adhesion properties of SCARA5 and its potential for increasing recombinant protein production in mammalian cells by improving the cell viability and culture properties of the cells as well as making it easy to select cells with high expression.
4. To characterize the expression pattern and *in vivo* function of the novel class A scavenger receptor SCARA5 by generating specific antibodies and a SCARA5 knockout mouse line.

3 METHODS

The methods used in the included papers (I-IV) are described in detail in the respective Materials and Methods sections.

Surface plasmon resonance [I]

A biochemical method to measure the equilibrium dissociation constant (K_D) of an analyte and its ligand.

Production and purification of proteins [I-IV]

Proteins were produced either in *E. coli* [I, IV] or in mammalian cells [I-IV]. Conventional chromatography methods (anion/cation exchange chromatography, size exclusion chromatography) [II], affinity tags (Glutathione S-transferase (GST), polyhistidine, strep) [I, III, IV] or self-generated antigen coupled affinity matrixes [I, IV] were used for purification.

Generation of antibodies [I, IV]

GST and SCARA5 specific polyclonal antibodies were generated by immunizing rabbits with different fragments of SCARA5 either as GST fusion proteins or with polyhistidine tag.

Ligand-Binding studies [I, II, IV]

Several ligand-binding studies were used. In some cases purified proteins (soluble MARCO, SRCR domain) were coated on surfaces and overlaid with different ligand solutions to study the binding (BIAcore, phage display) [I]. In other types of assays cells expressing the protein of interest (transfected Chinese hamster ovary (CHO) cells, primary cells) were incubated with the ligand solution (Fusion peptide, fluorescently labeled bacteria, LPS and AcLDL) [I, II, IV].

Isolation of primary cells [IV]

Different immune cell populations were isolated with magnetic dynabeads coupled with cell-specific antibodies. Primary fibroblasts were isolated from a cell suspension from which the macrophages were removed with dynabeads. After overnight plating, only fibroblasts stay attached on the cell culture plastic.

Western blotting [III, IV]

A method to detect and determine the size of a protein electrophoresed on a gel separating proteins based on their molecular weight. The proteins on the gels were then transferred (blotted) to a membrane where the protein of interest was localized using specific antibodies against it. Here it was used to detect proteins in different tissue lysates [IV], different fibroblast/macrophage cell lines [IV], and transfected cells [III]

Northern Blotting [IV]

A method to detect and determine the size of a messenger RNA first size-fractionated on a gel and then blotted on a membrane. The RNA of interest was then localized using a labeled probe specific for the RNA molecule.

Southern Blotting [IV]

A method to detect a specific genomic DNA fragment first size-fractionated on a gel and then blotted on a membrane. The DNA of interest was then localized using a labeled probe specific for the DNA molecule.

Immunostaining [I-IV]

Immunofluorescence stainings were made to detect either the cell-surface proteins [I, II, IV] or the total proteins on permeabilized cells with MARCO, SCARA5, F-actin or Crb2 specific antibodies and fluorescently labeled secondary antibodies [I-IV].

Histology and immunohistochemistry [IV]

Light microscopy was used to examine the histology of different organs of experimental animals on paraffin embedded tissue sections stained with SCARA5 specific antibodies and/or hematoxylin and eosin. Several other cell type markers were similarly localized in the tissues with specific antibodies. Fresh frozen tissue sections were stained with SCARA5, CD31, PDGFR α , α -smooth muscle actin (ASMA), F4/80, CD11b, fibroblast specific protein (FSP), and vimentin to identify the cell type expressing SCARA5 and analyzed under fluorescence or confocal microscope.

Flow cytometry or FACS (Fluorescence activated cell sorter) [III]

A method to quantify different cell populations based on the cell shape, size, and expression of different molecules. Here the proportion of SCARA5 positive cells in the cell suspension before and after the selection step was analyzed.

Crystallography [II]

Hanging drop vapor diffusion method was used to crystallize the purified monomeric and dimeric SRCR domain of MARCO. After indexing, integration, and scaling of the x-ray diffraction data, the first structure solutions were generated by molecular replacement followed by several rounds of iterative model building and refinement.

Chemical cross-linking [II]

In order to determine the oligomerization state of purified SRCR domains of MARCO, the proteins were mixed with amine-to-amine reactive cross-linker dithiobis(succinimidyl propionate) (DSP) and analyzed on SDS-PAGE.

Generation of recombinant protein producing cell lines [III, IV]

In order to produce large quantities of functional mammalian proteins (different soluble forms of the extracellular part of SCARA5 and globular subdomains 1-3 (LN3G) of the mouse laminin α 3 chain) HEK-293 EBNA cells were transfected with constructs expressing the protein of interest. When using a conventional selection method, the cells with incorporated constructs were selected based on the antibiotics resistance conferred by the expression construct [III, IV]. In paper III, a new method to select the cells based on the SCARA5 adhesion is described.

Cell adhesion assay [II, III]

Cell adhesion assay was performed on cells expressing normal MARCO and different mutants of the SRCR domain [II]. In these assays the cells were plated on surfaces coated with gelatin, polyinosinic acid (Poly I), or polylysine in media with or without

Ca²⁺ and Mg²⁺. All the cells adhere to polylysine while only MARCO expressing cells adhere to gelatin and poly I. The cells remaining on the surfaces after washes were quantified by F-actin staining. In the adhesion assays with SCARA5 expressing cells [III], the cells were plated on tissue culture plastic. Cells remaining on the plates after washes were quantified by crystal violet staining. In some assays the cells were treated with EDTA or poly I to block the adhesion through SCARA5.

Generation of expression constructs [I-IV]

Conventional molecular biology methods were used to clone constructs for expression of GST-phage peptide [I], MARCO SRCR domain mutants [I, II], LN3G-protein [III], green fluorescent protein (GFP) and SCARA5 for selection [III], intra and extracellular part of SCARA5 in bacteria and mammalian cells for antibody production and full length SCARA5 for binding assays [IV].

Phage display screen [I]

A phage library solution was overlaid onto plates coated with soluble MARCO. The wells were washed and bound phages were used to infect competent K91kan *E.coli*. The procedure was repeated three additional times and finally randomly picked, phage-infected *E.coli* clones were sequenced to identify the peptides displayed by the phages.

Animals and animal experiments [IV]

A SCARA5 gene knockout construct was designed so that the transmembrane domain of the protein was deleted. The construct was used to generate a chimeric SCARA5 knockout mouse line. The mice were backcrossed to C57BL6 strain for 10 generations. Some SCARA5 knockout mice were mated with PDGFR α knock-in mice (B6.129S4-*Pdgfr* α ^{tm11(EGFP)Sor/J}) to generate knockout mice with GFP protein expression in the nucleus of PDGFR α expressing cells. These mice were used to confirm the coexpression of SCARA5 and PDGFR α in certain fibroblasts. Material for histological analysis and different stainings as well as for isolation of primary cells was collected from mice with different ages.

RT-PCR and qPCR [IV]

Total RNA was isolated from different isolated immune cells and C3H 10T1/2 cell line. RT-PCR reaction was performed to produce a cDNA library from the RNA. Similar cDNA libraries generated from different tissues were purchased from BD Biosciences. The cDNA libraries were used in qPCR to detect the amount of different class A scavenger receptors (SCARA1 to 5) in the immune cells or to detect the presence of SCARA5 mRNA in different tissues.

Ethical considerations [I-IV]

All the experimental studies were conducted according to regulations related to handling of laboratory animals and approved by local ethical committees.

Statistical analysis [III]

Data were analyzed either with student t-test.

4 RESULTS

Following the initial cloning of class A scavenger receptor MARCO (SCARA2) (Elomaa et al., 1995), several studies have shown that MARCO is a pattern recognition molecule recognizing various ligands ranging from modified/unmodified self-molecules to environmental particles and different bacteria and their cell wall components. The broad ligand repertoire can be seen as an indication of MARCO's role in tissue homeostasis and as a molecule involved in innate immunity and defense against bacterial infections (Arredouani et al., 2004; Bin et al., 2003; Elomaa et al., 1995; Gordon, 2002; Krieger and Herz, 1994). In the present thesis project, further evidence was provided on ligand-binding kinetics as well as the mode of binding to the C-terminal SRCR domain of MARCO (Papers I and II).

While the role of the SRCR domain in MARCO's ligand-binding function was studied, we identified a structurally similar previously unknown protein SCARA5 using mining of publicly available genome sequences. Our initial ligand-binding studies with SCARA5 showed that similar to MARCO, the novel receptor was able to bind bacteria. However, SCARA5 exhibited very low affinity to AcLDL. This notion was used as a tool to further study the function of MARCO SRCR domain (Papers I and II).

To gain more understanding of the functional differences and similarities of MARCO and SCARA5, a mouse line deficient for the *Scara5* gene was generated. By using this mouse model, SCARA5's tissue expression pattern and its role in tissue homeostasis and development of a connective tissue disease resembling some autoimmune diseases seen in humans was elucidated (Paper IV). As SCARA5 expression was found to be restricted to a subpopulation of fibroblasts and we had made a notion that similar to MARCO (Papers II and IV), SCARA5 adheres strongly to surfaces coated with gelatin, SCARA5 expression was used to improve the cell culture properties and protein production in mammalian cells (Paper III).

4.1 THE ROLE OF MARCO SRCR DOMAIN IN LIGAND-BINDING ELUCIDATED BY PHAGE DISPLAY SCREENING AND ACETYLATED LDL BINDING (PAPER I)

Evidence that MARCO SRCR domain has a functional role was observed when cells transfected with a truncated form of MARCO lacking the SRCR domain failed to induce typical morphological changes (Pikkarainen et al., 1999) and exhibited very low bacteria binding as compared to the full length MARCO (Brannstrom et al., 2002). In order to further study the ligand binding to MARCO, a mammalian expression system to produce soluble forms of the whole extracellular region of MARCO (sMARCO) as well as the SRCR domain was established. It was shown that functional proteins were generated and binding of bacteria and LPS could be detected in surfaces coated with sMARCO (Sankala et al., 2002). In Paper I, we sought to further characterize the biochemical binding properties of various ligands to sMARCO as well as identify possible new ligands with the help of a phage display screen.

First, a surface plasmon resonance (BIAcore) based system to study the ligand binding to sMARCO was established. We could show avid binding of both Gram-positive and -negative bacteria cell wall components LTA and LPS. Even stronger binding could be seen for another polyanionic molecule poly I, which is often used as a

blocking reagent when studying the function of scavenger receptors. Most of the identified ligands binding to scavenger receptors are polyanionic in nature (Gordon, 2002). However, negative charge is not the only attribute behind binding, since no binding to sMARCO was detected with heparin.

Next, a phage display screen was performed with sMARCO to identify possible new ligands that could be studied on BIAcore. After several rounds of screening, five peptide sequences were obtained, out of which two showed clear prevalence. Only these two peptides, VRWGSFFAAWL and RLNWAWLSY, were studied further. Indeed, the VRWGSFFAAWL peptide showed strong binding to sMARCO also in a BIAcore setting. Unexpectedly, instead of being anionic in nature both of the peptides were composed of mostly hydrophobic residues.

In the next set of experiments, the phage-peptide binding site in MARCO was localized to the SRCR domain. First, no binding of either of the phage-peptides could be detected on transfected cells expressing on their cell surface a truncated form of MARCO lacking the SRCR domain. In contrast, cells expressing a full-length MARCO showed avid binding. Second, this result could be confirmed by overlaying SRCR domain, sMARCO, control proteins BSA and recombinant Nephrin coated plates with the VRWGSFFAAWL phage. Only wells with the SRCR domain and sMARCO showed any binding. All the studies described above were conducted with the phage peptides. However, similar results were obtained when the experiments were conducted with a recombinant GST coupled fusion peptide or a synthetic peptide. Furthermore, these additional tests suggested that both peptides bind to the same site in the SRCR domain. However, the first 17 residues extending to the SRCR domain were not sufficient to restore binding to the peptide even though such a protein showed avid binding to bacteria (Brannstrom et al., 2002).

In one set of the experiments, the human version of MARCO was found to bind the GST-peptide; the amino acid sequence identity between the human and mouse SRCR domains is 78 %. In contrast, there was no binding to full-length SCARA5. The SRCR domain of SCARA5 shares 45 % sequence identity with MARCO's SRCR domain (Figure 3). However, when the SRCR domain of SCARA5 was totally or partially replaced by sequences of MARCO SRCR domain, an IW mutant with MARCO residues 423-481 and NC mutant with residues 423-507, the mutated SCARA5 protein was able to bind the GST-peptide fusion protein. This indicates that the peptide-binding site of MARCO SRCR domain resides within the sequence 423-481 (Figure 3).

```

mMARCO 420 FORVRIM--GGTNRGRAEVYNNNEWGTCDDDDWDNNDATVFCRMLGYSRGRAL---SSYG 474
              F  +R++  G  +GR EV+++  WGT+CDD WD  D  V CRMLG+  +  + +G
mSCARA5 386 FTMIRLVNGSGPHQGRVEVFHRRRWGTVCDGWDKKGDDVVCRLGFHGVVEVYRTARFG 445

475 GGSGNIWLDNVNCRGTENSLWDCSKNSWGNHNCVHNEDAGVECS 518
              G+G IW+D+VNC+GTE S+  C  WG  NC H EDAGV C+
446 QGTGRIWMDDVNCKGTESSIFHCQFSKWGVTNCGHAEDAGVTCTVP 491
  
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Figure 3. Amino acid sequence alignment of MARCO and SCARA5 SRCR domains. The mouse sequences were aligned with the BLAST program at NCBI database <http://www.ncbi.nlm.nih.gov/>. The sequences showed 45 % sequence identity. The amino acid residues from the MARCO sequence included in an IW mutant of SCARA5 are underlined with black line. In a similar fashion, red line denotes the amino acid residues mutated in an NC mutant.

As mentioned earlier, we found that intact SCARA5 binds AcLDL very poorly. Therefore, we also tested the SCARA5 mutants with the whole MARCO SRCR domain and fragments of MARCO (IW and NC mutants) for AcLDL binding. Yet again, we could prove that the MARCO SRCR domain is important for ligand-binding, since the mutant with an intact MARCO SRCR domain bound strongly to AcLDL (Figure 4). Interestingly, the two latter mutants IW and NC were unable to bind AcLDL indicating that the last 11 residues of MARCO are required for the binding (Figure 3). However, AcLDL seems to bind to a different location on MARCO SRCR domain than the isolated phage peptide. The SRCR domain of SR-AI had previously not been shown to have any function, but when we tested it in a similar manner, we could see avid binding (unpublished data, Figure 4).

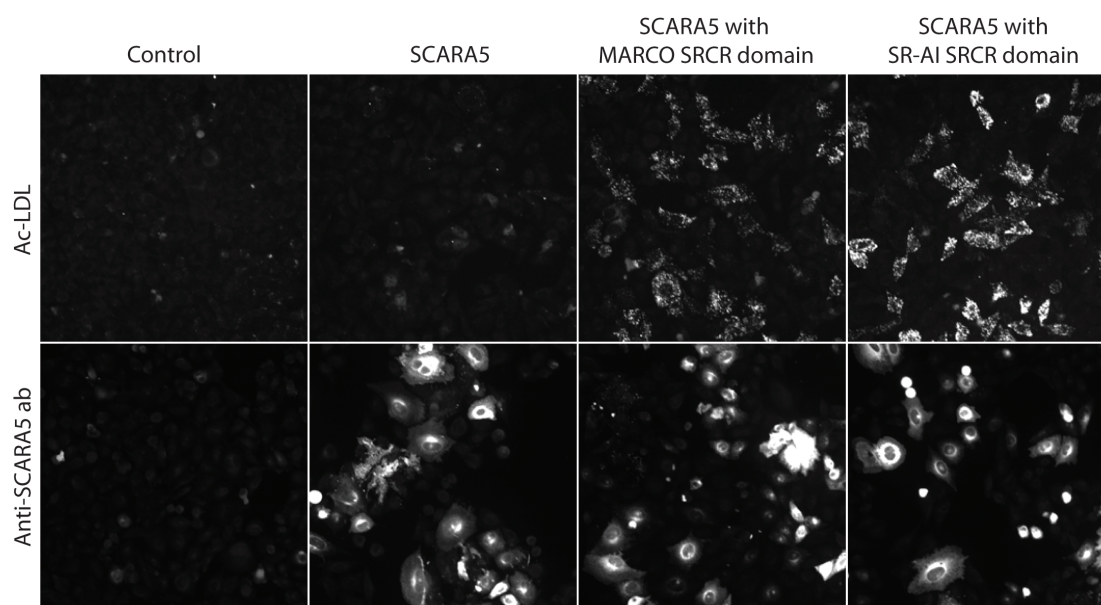


Figure 4. Binding of AcLDL to cells expressing SCARA5 and its variants with SRCR domains from MARCO and SR-AI. CHO cells were transiently transfected with a control plasmid and plasmids expressing full-length SCARA5 and mutants with SRCR domains from MARCO or SR-AI. The binding of AcLDL (white dots) to these cells was tested. Top row, control cells and SCARA5 transfected cells exhibit hardly any binding of AcLDL whereas avid binding can be detected on cells expressing SCARA5 mutants with MARCO and SR-AI SRCR domain. Bottom row, the transfection efficiency and protein expression was confirmed by immunostaining of the cells with antibodies recognizing the intracellular part of SCARA5.

The purpose of the phage display screen was to search for new potential MARCO ligands. Interestingly, when the sequences of the identified peptides were used to screen protein databases, the closest sequence match could be found in the complement component C4. When the interaction between C4 and MARCO was tested, we could detect weak binding of C4b and C4d to sMARCO and cells expressing full-length MARCO. However, the binding could not be blocked by the GST-peptide demonstrating that if the interaction is true, C4 fragments and the peptide bind to distinct sites on MARCO. Furthermore, tissue-staining attempts to find endogenous ligands with antibodies recognizing the peptide sequence were made to no avail.

4.2 CRYSTAL STRUCTURE REVEALS MULTIPLE BINDING SITES IN THE MARCO SRCR DOMAIN (PAPER II)

As was evident from previous studies, most identified ligands of mouse MARCO bind to the SRCR domain (Brannstrom et al., 2002; Chen et al., 2006; Pikkariainen et al., 1999; Sankala et al., 2002). In order to gain better understanding of the ligand-binding properties of the domain we sought to solve the crystal structure of the domain. The protein was produced using previously established mammalian cells stably expressing the mouse MARCO SRCR domain starting from the amino acid residue Q421 (Figure 3) (Sankala et al., 2002). The protein contained no purification tags and therefore the purification was achieved using conventional ion-exchange and size exclusion chromatography methods. Based on the size exclusion chromatography and chemical cross-linking studies, the protein could be purified in a monomeric and dimeric form (Figure 5).

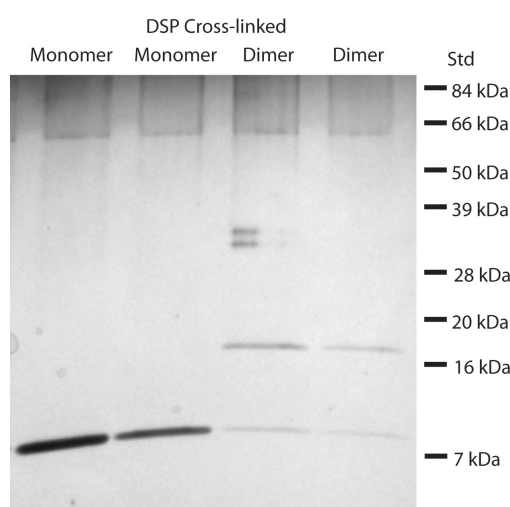


Figure 5. Chemical cross-linking of different forms of purified MARCO SRCR domains. The MARCO SRCR domain could be separated in two distinct pools during anion exchange chromatography. These pools had different retention times on the size exclusion chromatography indicating the presence of monomeric and dimeric forms of the protein. This could be confirmed when the proteins were chemically cross-linked with amine reactive dithiobis(succinimidyl propionate) (DSP) and size-separated on an SDS-PAGE gel. The sample order in the silver stained gel is indicated above the gel. Based on the molecular weight standard sizes, the protein was purified as monomer and dimer. The expected molecular weight of a monomeric MARCO SRCR domain is 11 kDa.

The purified proteins were crystallized using a hanging drop vapor diffusion method. After some optimization, good quality crystals were obtained and used to collect diffraction data (Figure 6). Relatively good quality data was collected up to 1.78 Å and 1.77 Å for the monomeric and dimeric forms of the SRCR domain, respectively. Both structures were solved by molecular replacement and after the final refinement both structures had good stereochemistry and the R-factors were within acceptable range indicating that the structures were correct.

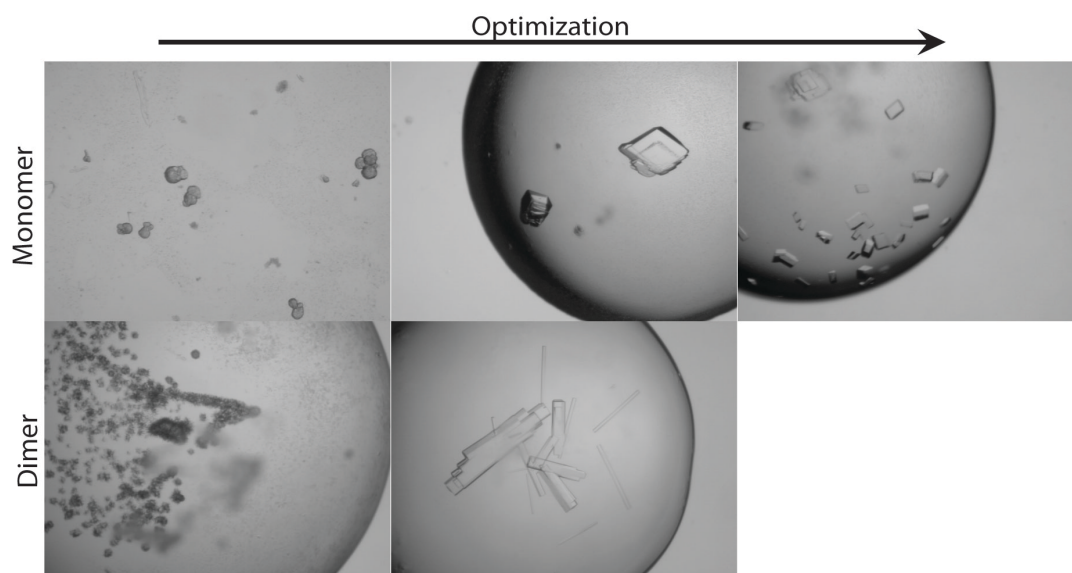


Figure 6. Crystallization of the monomeric and dimeric form of MARCO SRCR domain. After optimization of the crystallization conditions, monoclinic crystals were obtained of the monomeric form of the protein (upper panel). The crystals of the dimeric form showed triclinic appearance (lower panel).

The monomeric MARCO SRCR domain structure has a compact globular fold (~ 30 Å in diameter) with a single α -helix wrapped from one side by a twisted β -sheet composed of six (A-F) antiparallel β -strands and a long loop arching over to the other side. Two disulfide bonds stabilize the long loop region. A third disulfide bond is connecting the C-terminal end of the protein to the α -helix. The β -strands A-C in the N-terminus are held in place mainly by hydrophobic contacts. Two Mg^{2+} ions were modeled into the structure (Figure 7 A-B).

The dimeric form of the purified MARCO SRCR domain was relatively stable, but it dissociated in solutions containing trace amounts of nonpolar solvents. This phenomenon could be explained by the dimeric structure that formed through β -strand swapping of the first N-terminal β -strands (A) in each monomeric domain structure. Strikingly, a large ($\sim 26 \times 15$ Å) eight-stranded antiparallel β -sheet is formed due to the β -strands swapping. The dimer had three bound sulfate ions in the structure (Figure 7C).

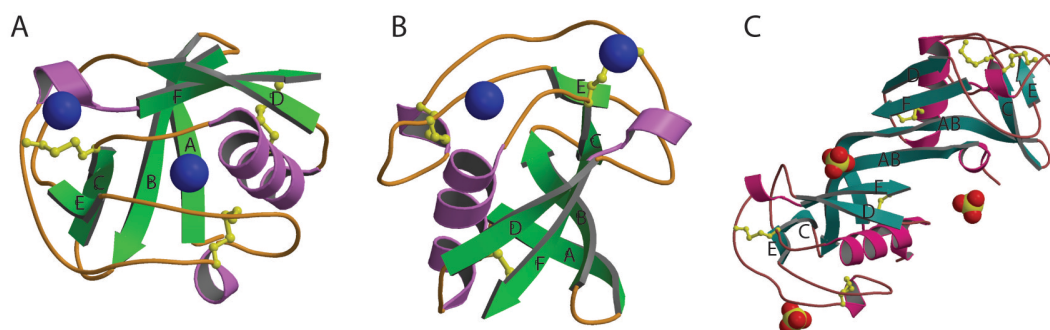


Figure 7. Monomeric and dimeric structures of the MARCO SRCR domain. A and B, the monomeric structure is presented in two different orientations. The β -strands A-F are presented in green, helices are colored in pink. Disulfide bonds are shown in yellow. Two bound Mg^{2+} ions are shown as blue spheres. C, a similar representation of the dimeric structure. Three sulfate ions are shown in yellow-red.

In order to detect putative domain motions, the monomeric structure coordinates were submitted to the Dynamite server (Barrett et al., 2004), which returned possible modes of dynamics. The covariance lines presented in Figure 8A highlight regions that will move together. From the stereo image it can be seen that the β -turns AB and BC, the end of the α -helix together with the β -strands D and F as well as the long loop region move as separate units. The porcupine stereo image seen in Figure 8B shows the direction and magnitude of the movements.

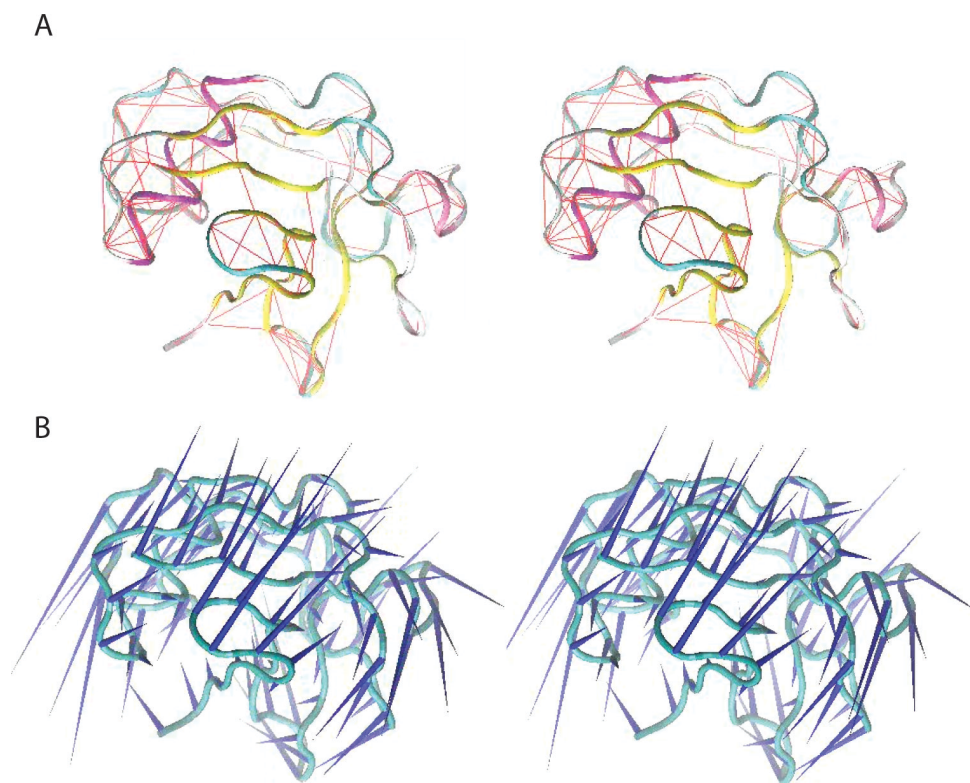


Figure 8. Stereo representation of possible modes of molecular movements in the monomeric MARCO SRCR domain structure. A, a stereo image of the monomeric MARCO SRCR domain with covariance lines (red) highlighting the regions that move together. B, porcupine representation of the principal motion in stereo. Each cone (blue) renders the magnitude and direction of the motion.

Since the ligands that bind to scavenger receptors generally are anionic in nature, we expected to find positively charged regions in the surface of the structure. Indeed, the surfaces around the β -sheet structures in both the monomeric and dimeric structures have clearly a positive electrostatic potential. The area is especially prominent in the dimeric structure. Importantly, this region contains three linear RXR motifs and two structural RER motifs that have previously been shown to be important for bacteria binding (Brannstrom et al., 2002). To our surprise, a roughly as large negative cluster ($\sim 20 \times 20 \text{ \AA}$) as the positive cluster was found in the structure. The cluster is located on the opposite end of the molecule at the long loop area. Some of the negative charge is neutralized by bound Mg^{2+} ions in the monomeric structure. In the dimeric structure some residues of a symmetry related molecule is making contacts with the aspartates in the negative cluster.

The structural fold of the monomeric structure is very similar to the other published SRCR domain structures, such as those of hepsin, M2BP and CD5 (Hohenester et al., 1999; Rodamilans et al., 2007; Somoza et al., 2003). The SRCR

domains of M2BP and MARCO belong to class A SRCR domains and the structures differ only in the lengths of loops AB and DE. In the structure of class B SRCR domain of CD5 there is an additional disulfide bond that stabilizes the β -strand B in place. The SRCR domain of Hepsin has slightly different disulfide bond pattern. The surface electrostatic potentials vary dramatically in each of these structures. This is also true for a modeled structure of SR-A, in which the negative cluster is slightly smaller and closely flanked by a positive cluster. Other proteins sharing parts of the fold include toxin II from the scorpion *Androctonus australis* and antibacterial protein sapecin. These structures align well with the α -helix and β -strands D and F of MARCO SRCR domain, the region indicated in ligand binding.

Next, the importance of the positively charged cluster in the MARCO SRCR domain for cell adhesion to gelatin-coated surfaces was tested by a set of constructs where different combinations of the arginines were mutated. The results indicate that the adhesion is achieved co-operatively through several arginines, since none of the single mutations affected the adhesion, whereas several of the combinatorial mutants lost the adhesion completely. Many of the same mutants also showed low binding of AcLDL. In another set of experiments the mutations were directed to the negative cluster. Strikingly, double mutations of D447 and D448, as well as a single mutation of E511 showed very low adhesion to gelatin, did not bind to AcLDL, and failed to induce typical morphological changes to the cells (Pikkarainen et al., 1999). Interestingly, a combination of these mutations reversed the affects completely. Since the residues in the acidic cluster of MARCO SRCR domain participated in ion binding, the adhesion to gelatin-coated surfaces was tested in the presence and absence of divalent ions. Indeed, the experiments showed that the presence of Ca^{2+} was required. Similar results were obtained in the presence of Mn^{2+} but not in the presence of Mg^{2+} . Interestingly, the acidic cluster's triple mutant did not require ions for the adhesion. When cell adhesion of the mutants in both groups were tested on poly I coated surfaces, similar results were obtained.

We found earlier that SCARA5 has low affinity for the prototypical scavenger receptor ligand AcLDL (Figure 4). Similar results have been reported for human MARCO (Elshourbagy et al., 2000), which indicates that small differences between the SRCR domain sequences affect the binding to AcLDL. However, arginines do not seem to play such a crucial role since only arginines 422 and 466 are different between the 74% identical human and mouse sequences. To test which residues are required for the binding, we generated chimeric proteins where the mouse SRCR domain was replaced by the whole human SRCR domain or parts of it. First, we found that both human MARCO and the mouse MARCO chimera with human SRCR domain did exhibit some binding of AcLDL. Regardless, when the human SRCR domain sequence was changed back to the mouse sequence at the end of the molecule (residues 496-518) the same way as was done for SCARA5 earlier (IW and NC mutants), increased binding of AcLDL was observed. The binding was even stronger when residues 453-518 were changed into mouse sequence. In contrast, a chimera with residues 449-473 from mouse MARCO had no effect on AcLDL binding indicating that the residues affecting the binding reside between residues 473-518.

4.3 SCARA5 EXPRESSION IMPROVES VIABILITY OF MAMMALIAN CELLS IN SERUM-FREE CULTURE CONDITIONS AND CAN BE USED AS AN ALTERNATIVE TO ANTIBIOTICS FOR SELECTING STABLE PROTEIN-PRODUCING CELL LINES (PAPER III)

Selected HEK-293 EBNA cells expressing the globular subdomains 1-3 (LN3G) of the mouse laminin $\alpha 3$ chain showed very low adhesion to cell culture plastic and did not survive in the serum-free conditions during protein production. Since the produced laminin fragment represents the main adhesive site of laminin-332, we suspected that the produced recombinant protein is disrupting the normal cell adhesion by blocking the integrin interaction (Miner and Yurchenco, 2004). As opposed to integrin-laminin mediated adhesion, studies I and II showed that MARCO mediates strong adhesion to surfaces coated with gelatin or poly I, whereas regular HEK-293 or CHO cells showed very low affinity to these surfaces. In addition, we had observed that SCARA5 also mediates strong adhesion to cell culture plastic and gelatin coated surfaces.

Therefore, in an attempt to circumvent the adhesion problem, the LN3G expressing cells were co-transfected with SCARA5. Indeed, a significantly higher adhesion on cell culture plastic was observed on cells expressing SCARA5 and the protein production was successful. In fact, SCARA5 positive cells produced up to twofold higher levels of recombinant proteins than the parental cells. Furthermore, it was evident that SCARA5 positive cells showed increased cell viability in the serum-free culture conditions further facilitating the protein production. In further tests we could show that SCARA5-mediated adhesion was cation-independent and could be blocked by poly I treatment.

Prompted by these results, we sought to explore SCARA5 adhesion for selecting stable clones for protein production. For the selection, we chose to use gelatin coated surfaces because the most often used cells lines for protein production such as HEK-293 and CHO cells were unable to adhere to these surfaces. Other tested cell types (C3H 10T1/12, NIH 3T3, COS-1, HeLa, and L929) adhered on their own to gelatin coated surfaces. To be able to monitor the selection process, we generated a GFP-IRES-SCARA5 selection construct that produces a single mRNA for both GFP and SCARA5. When the number of green cells were quantified by flow cytometry before and after the selection procedure, up to 60-fold increase could be detected in the cell population left after selection. Similar results could be seen when the cells were left adherent and the expression of GFP and SCARA5 was examined by immunofluorescence.

4.4 DEFICIENCY OF FIBROBLAST EXPRESSED SCARA5 IN MICE CAUSES AUTOIMMUNE DISEASE -LIKE PHENOTYPE (PAPER IV)

A novel class A scavenger receptor was identified while doing data mining in the NCBI public database with MARCO SRCR domain sequence. The sequence was well conserved between species and had a very similar domain structure as both MARCO and SR-A that had previously been studied in our laboratory. To study if this new receptor had similar immune related functions as MARCO and SR-A, we generated gene knockout mice, cloned the cDNA, and studied its expression pattern in different tissues at both the RNA and protein level by several methods. During the progress of

this study, identification of the same protein, termed Tesr or SCARA5, was reported (Jiang et al., 2006; Sarraj et al., 2005).

In our initial screen by Northern blot and RT-PCR analysis, we found SCARA5 mRNA in almost all the tissues. The broad tissue expression pattern at protein level was confirmed by Western blot analysis from different tissue lysates. The Western blot analysis indicated that SCARA5 exists *in vivo* as a trimeric molecule. Each subunit has apparent molecular weight of ~90 kDa, which suggests that the protein is heavily glycosylated.

Based on structural similarity to MARCO and SR-A, we expected to find the protein in macrophages. However, the initial immunohistochemical stainings of different tissues suggested otherwise. Co-localization studies and confocal microscopy with markers staining endothelial cells (CD31), macrophages (F4/80, CD11b), and different fibroblasts populations (ASMA, vimentin, PDGFR α , FSP) revealed that SCARA5 expression is restricted to a subpopulation of fibroblasts. Notably, the expression overlapped mostly with vimentin and PDGFR α (Figure 9), which are highly expressed in fibroblasts.

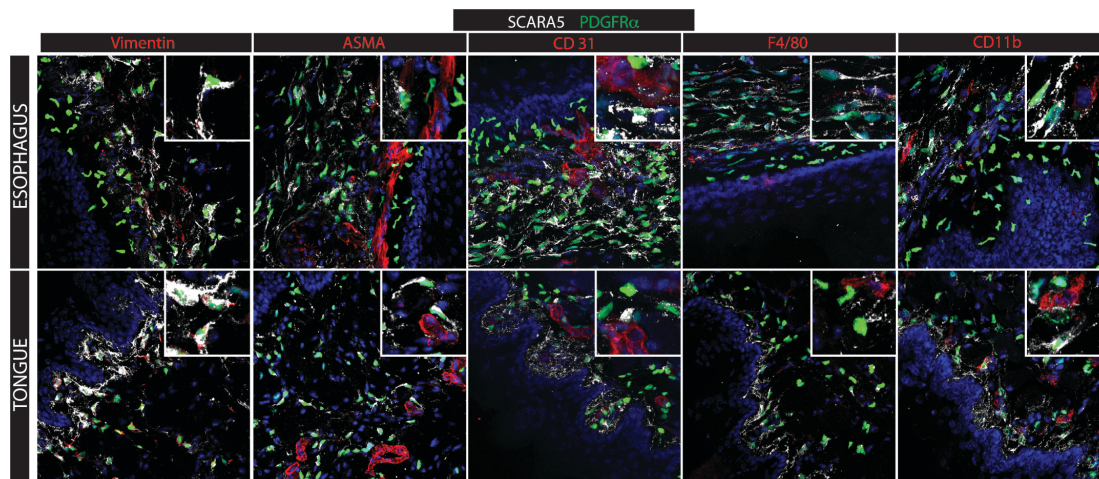


Figure 9. SCARA5 is co-expressed in vimentin and PDGFR α -positive fibroblasts. Immunofluorescence staining and confocal microscopy of esophageal (upper row) and tongue (lower row) tissue sections with antibodies recognizing SCARA5 (white), vimentin (red) first panel, ASMA (red) second panel, CD 31 (red) third panel, F4/80 (red) fourth panel and CD11b (red) fifth panel. PDGFR α expression is seen as green nuclear signal, because the sections were obtained from mice in which a nuclear localized H2B-eGFP fusion gene is expressed from the endogenous *Pdgfra* locus (Hamilton et al., 2003). Rest of the nuclei were stained with DAPI (blue). Indent, 2.7 x additional magnification. SCARA5 is co-expressed with vimentin and PDGFR α positive cells but not of those expressing ASMA, CD31, F4/80 or CD11b.

Additional expression of SCARA5 was found in the apical side of sertoli cells in testis epithelium and the epithelial cells of choroid plexus. Of note, SCARA5 positive cells in choroid plexus express PDGFR α (unpublished data) and vimentin expression is seen in the basal side of sertoli cells, while PDGFR α expression is located in the interstitial cells. Fibroblast expression of SCARA5 was also found in isolated primary fibroblasts and several fibroblast cell lines, whereas no expression was seen in macrophage cell lines. Furthermore, other sources of immune cells (whole blood, Spleen B- and T-cells, lymph node, peritoneal macrophages, dendritic cells, and bone marrow) showed no SCARA5 expression, even though they expressed other types of class A scavenger receptors.

It was previously shown that SCARA5 cells do not bind avidly to prototypic scavenger receptor ligand AcLDL (Figure 4) (Chen et al., 2006; Jiang et al., 2006). However, similar to MARCO and SR-A, SCARA5 was found to be an endocytic receptor binding strongly to *E.coli* and LPS, the outer cell wall component of Gram-negative bacteria.

In order to study the *in vivo* function of SCARA5, knockout mice were generated where the DNA sequence encoding the transmembrane region of the protein was deleted. The knockout mice were born with normal Mendelian distribution and showed no apparent dysfunctions at young age even though no functional SCARA5 protein could be detected. At around 6 months of age several SCARA5 deficient mice were found to have lymphocyte infiltrates in several organs. The inflammatory reactions were seen most prominently in the lung with severe lymphocyte infiltrates around pulmonary arteries, accumulation of Ig-producing plasma cells and macrophages and necrotic pseudogranulomatous nodules. Similar symptoms can be seen in several types of autoimmune conditions such as Sjögren's disease, SLE and rheumatoid arthritis. To support the notion that the symptoms seen in the SCARA5 deficient mice are of autoimmune disease –type, a significant number of the knockout mice were found to develop autoantibodies against nuclear proteins.

5 DISCUSSION

In this work we aimed to study the ligand-binding function of mouse MARCO and later on the physiological function of another related scavenger receptor SCARA5. Our finding of hydrophobic peptides binding to the SRCR domain of MARCO was surprising, since most scavenger receptor ligands identified so far such as Gram-negative and –positive bacteria are polyanionic in nature (Brannstrom et al., 2002; Elomaa et al., 1998). On the other hand, it should be noted that MARCO has been shown to bind diverse other molecules such as apoptotic cells, UGRP1, myramyl dipeptide (MPD) and environmental particles (Bin et al., 2003; Mukhopadhyay et al., 2011; Palecanda et al., 1999; Wermeling et al., 2007). In contrast to earlier cell-based studies where other cell surface molecules may affect the binding, we were able to utilize cell-free systems with soluble forms of MARCO to show binding of the major cell wall components of Gram-negative and –positive bacteria, LPS and LTA, respectively. However, these polyanions had much faster dissociation kinetics than the identified peptides or poly I, the commonly used polyanionic ligand-binding inhibitor of scavenger receptors. In contrast, there was no specific binding to another polyanionic molecule heparin.

In our database searches with the most enriched peptide sequence VRWGSFAAWL, most matches were from the transmembrane sequences of various proteins and were disregarded even keeping in mind that such sequences could be recognized e. g. on the surface of apoptotic cells. As a potential ligand for MARCO a nearly matching sequence GSYAAWL could be identified in another innate immunity effector molecule complement component C4. Indeed, we could detect binding to C4b and C4d. C4d is a physiological degradation fragment of C4b. These are the forms of C4 that act as opsonins once bound to the surface of a bacterium. Interestingly, the complement component iC3b, another opsonizing complement factor, was identified as a ligand binding to the SRCR domain of SR-AI (Goh et al., 2010). However, the true ligand corresponding to the peptide sequence still remains to be identified, since the binding of C4b could not be blocked by the peptide. Furthermore, a close examination of the C4d structure suggest that only the WL sequence is exposed indicating that the identified sequence in C4 is not participating to the binding (van den Elsen et al., 2002).

Even without identifying the true ligand corresponding to the peptide sequence, we wanted further characterize the ligand-binding site in MARCO. Rotary-shadowing microscopy showed that sMARCO used for phage-display screening is a rather long (~80 nm) rod-like molecule composed of a spacer region, a long collagenous domain and the C-terminal SRCR domain making it hard to pinpoint the ligand-binding site (Sankala et al., 2002). As for the other MARCO ligands identified earlier, our cell-studies with MARCO protein devoid of the SRCR domain showed that the isolated peptide binds to the SRCR domain. However, there was no binding to a bacteria-binding MARCO version extending 17 residues to the SRCR domain (Brannstrom et al., 2002; Elomaa et al., 1998). As a further confirmation, we could also show binding of the peptide to a purified SRCR domain. However, there was a clear difference between the number of phages bound to the SRCR domain and sMARCO indicating that there is a difference in the binding affinity. Interestingly, similar affinity differences were seen when bacteria binding was studied on surfaces coated with sMARCO and the SRCR domain (Sankala et al., 2002). Whether the difference is due

to avidity or difference in the way proteins are coated to the surface remains to be seen; when a single homotrimeric sMARCO with a long collagenous stalk and three SRCR domains participating to the binding is coated on a surface it is likely that the SRCR domains would still be readily available for binding (Sankala et al., 2002).

Further tests showed that the 74% identical human counterpart of the domain was also able to bind to the peptide, but there was no binding to a 45 % identical SRCR domain of SCARA5. When the SRCR domain sequence of SCARA5 was replaced by the mouse SRCR domain sequence or residues 423-481 (IW) and 423-507 (NC), the binding was restored indicating that the last 11 residues of the domain are not involved in peptide binding. In the crystal structure of the mouse MARCO SRCR domain the IW sequence encompasses most of the positively charged β -beta sheet structure (A-E) and the α -helix. NC sequence includes even the negatively charged long loop region, but lacks the last β -strand F residing in the middle of the β -sheet. Since SCARA5 binds normally very weakly to AcLDL, we tested the binding of AcLDL to these SCARA5 mutants as well. In contrast to the peptide binding, only the intact form of MARCO SRCR was able to bind AcLDL indicating that the negatively charged long loop region and/or β -strand F participate in binding. Comparison of the SCARA5 and MARCO sequences (GHAEDAGVTCTVP and VHNEDAGVECS, respectively) in the changed β -strand reveals only minor changes. However, a careful examination of the mouse MARCO SRCR structure revealed that mutation of E516 may alter the conformation of R431 and R468, which altogether normally form a structural RXR motif previously shown to be important for ligand binding (Brannstrom et al., 2002). As an indication how negatively charged molecules bind to this region, there is a sulfate ion coordinated to the arginines in the dimeric SRCR structure. An additional SCARA5 mutant was made, where the SRCR domain was from SR-AI. Since the ligand-binding region of SR-A was previously mapped to the end of the collagenous region (Doi et al., 1993) we were surprised to detect avid binding to AcLDL. This was the first evidence showing that the SRCR domain of SR-AI has a specific function, since the SR-AII splice variant lacking the SRCR domain has been shown to bind to the same ligands with only slightly different affinity (Peiser et al., 2000; Peiser et al., 2006). Supporting our finding, it was recently reported that the SRCR domain of SR-A is the binding site for iC3b (Goh et al., 2010).

After solving the crystal structure of mouse MARCO SRCR domain, it was evident that the domain contains a large positively charged cluster in the β -sheet region mentioned above. This large β -sheet region was especially prominent ($\sim 26 \times 15 \text{ \AA}$) in the dimeric structure and is likely the binding region for the polyanionic ligands of MARCO. For example, similar type of eight-stranded β -pleated sheet ($\sim 30 \times 12 \text{ \AA}$) represents the major peptide-binding groove in MHC molecules (Madden, 1995). More importantly, this region contains three linear and two structural RXR motifs implied in ligand binding (Brannstrom et al., 2002). Therefore, a set of arginine mutants was generated to further characterize the binding of AcLDL to this region. The binding turned out to be co-operative and only multiple mutations affecting both structural RXR motifs caused defective binding. However, arginines are not the only residues affecting AcLDL binding (E516 above), since the human SRCR domain with most arginines still in same places is not able to bind AcLDL (Elomaa et al., 1998). The mutants where the human sequence was changed to the mouse sequence suggest that the residues affecting the AcLDL binding lie between residues 474 and 518 and may confer major changes in the long loop region of the structure. Unexpectedly, this region

of the mouse MARCO SRCR structure turned out to have a net negative charge. Indeed, a double mutant of D447 and D448 or a single mutant of E511 within this negatively charged region abolished the AcLDL binding. One reason to this could be the fact that in the monomeric SRCR structure all of these residues are bound to a Mg^{2+} ion rendering the region more or less neutral. The charge change brought by the bound ion could also explain why a triple mutant of D447, D448 and E511 supported AcLDL binding. All and all, the residues of both the positively and negatively charged region participate in AcLDL binding. Interestingly, these are also the regions expected to display most molecular movements and include the peptide sequences in DMBT1 SRCR domain that participate in ligand binding (Bikker et al., 2013).

Apart from ligand binding, we found that the same positive and negative cluster mutants of mouse MARCO SRCR domain affect cell adhesion. We used as a substratum both gelatin and poly I coated surfaces where normal CHO cells adhere poorly. The results indicate that cell adhesion through MARCO SRCR domain is mediated by roughly the same regions in the SRCR domain as the ones affecting AcLDL binding. Interestingly, the multiple arginine mutant as well as the singular negative cluster mutants also abolished the morphological changes with dendritic processes typically seen for MARCO transfected cells (Pikkarainen et al., 1999). Yet again, this was not the case for the triple mutant of D447, D448 and E511. These findings led us to also test if the depletion of divalent cations affects the adhesion of MARCO transfected cells to these surfaces. As maybe expected, the negative cluster triple mutant was not affected, but normal MARCO mediated adhesion could only be restored if Ca^{2+} , Mn^{2+} or in some degree Mg^{2+} was present in the cell culture media.

When we saw lower binding of the phage peptide to the monomeric SRCR protein than to the trimeric sMARCO with three SRCR domains in each molecule we touched the subject of avidity. Similar effect could also be seen by the need of multiple arginines when binding to AcLDL. In fact, these studies were made with cells expressing trimeric MARCO molecules, so each single mutation needs to be multiplied by three; in essence a single arginine mutation with no effect corresponds to three mutated residues in a trimeric molecule whereas there are already nine mutated residues in a triple mutant. Keeping this in mind it is quite striking that single mutations such as E511 or E516 would have such a dramatic effect on ligand binding. However, when the overall structural changes caused by these mutations are examined it can be seen that E511 affects the binding of ions and thereby changes the charge of the whole region and E516 destabilizes the orientation of two arginine residues and therefore actually counts as a triple mutant. Another feature affecting the avidity could come from the dimeric structure that is essentially a β -strand-swapped form of two monomers. For example in the case of diphtheria toxin domain-swapping is induced by ligand binding (Louie et al., 1997). Thus, it is easy to envision that the SRCR domains (diameter ~ 3 nm) from separate MARCO molecules swap domains in order to create oligomerized forms of MARCO with multiple binding sites to support the binding of whole bacteria (diameter 0.2-2.0 μ m) or large macromolecules such as AcLDL (diameter ~ 25 nm) (Coresh et al., 1993). To support this notion, sMARCO molecules were often detected as oligomers in the rotary-shadowing images (Sankala et al., 2002). It is interesting to note here the MARCO mutant extending 17 residues to the SRCR domain reported to bind bacteria still has left all the residues involved in the domain-swapping in the dimeric structure and is therefore still able to form oligomers (Brannstrom et al., 2002).

As discussed earlier, MARCO supported cell adhesion to gelatin and poly I coated surfaces. Furthermore, the adhesion was found to require residues involved in the ion binding within the negative cluster of the SRCR domain structure and the presence of Ca^{2+} , Mn^{2+} or in some extent Mg^{2+} . When compared, all the residues involved in the ion binding are conserved between MARCO, SR-A and SCARA5, indicating that also SR-A and SCARA5 would function the same. Indeed, similar to MARCO both receptors show strong adhesion to tissue culture plastic but the adhesion was shown to be cation-independent (Fraser et al., 1993). Here it is important to note that the adhesion experiments with MARCO were made in a different manner; MARCO cells were incubated with a chelating agent in the suspension prior to plating to a gelatin coated surface while cell culture plastic adherent SR-A and SCARA5 cells were used. In the latter case, the ion supporting cell adhesion might be inaccessible to the chelating agent or there is a specific ion-dependent contact between MARCO and gelatin, which as a ligand differs dramatically from tissue culture plastic. Interestingly, SR-A mediated adhesion to cell culture plastic seems to block the bacteria/AcLDL - binding capacity of the receptor while cells plated on bacteriological plastic show avid binding (Peiser et al., 2000). Regardless of the adhesion mechanism, we found that SCARA5 supported adhesion increased the cell viability of cells used for protein production in serum-free culture conditions. This is an important feature, which can reduce protein production costs dramatically since the same cells can be harvested several times. In combination with this we could also see an increase in the yield of the produced recombinant protein LN3G. In our hands, LN3G-producing cells showed extremely low cell adhesion making it impossible to work with the cells. It can therefore be postulated that SCARA5 expression can be used to rescue the expression of other proteins causing similar type of adhesion problems as LN3G. We also exploited the SCARA5 mediated adhesion for clonal selection of stable protein-producing mammalian cell lines as an alternative to biohazardous and relatively expensive antibiotics. Reducing the costs is one thing, but it is also important to reduce the use of antibiotics from the global health perspective where multiresistant bacteria causing severe infections are a more and more common trade (Levy and Marshall, 2004). The method is also very simple and does not rely on specialized equipment needed in many traditionally used techniques (DeMaria et al., 2007; Sleiman et al., 2008).

Our results on SCARA5 provide new knowledge about fibroblasts. These cells have traditionally been considered as relatively inert cell population of connective tissue responsible for giving support to surrounding tissues by producing extracellular matrix (ECM) (Sorrell and Caplan, 2009). Thus finding a bacteria- and LPS-internalizing scavenger receptor SCARA5 on fibroblasts instead of macrophages, and an autoimmune disease like phenotype in knockout mice is intriguing as it suggests hitherto unknown properties for fibroblasts in tissue homeostasis. This data also contrasts another *in situ* hybridization-based study, where SCARA5 was localized to epithelial cells, that we could only confirm for the epithelial cells of choroid plexus and testis seminiferous tubules (Jiang et al., 2006; Sarraj et al., 2005).

In line with our present data, some other recent studies have described fibroblasts as a diverse cell population mediating important stromal cell – immune cell interactions which regulate the switch from acute inflammation to adaptive immunity and tissue repair (Buckley et al., 2001; Roozendaal and Mebius, 2011). Indeed, fibroblasts have been shown to express various chemokines and CD40, which regulates the infiltrated

hematopoietic cells via CD40 ligand (L) interaction (Brouty-Boye et al., 2000). Even a circulating antigen-presenting fibroblast population, fibrocytes, has been identified (Bucala et al., 1994; Chesney et al., 1997). Our results indicated that SCARA5 is only expressed by a subpopulation of fibroblasts (PDGFR α and vimentin positive, mostly FSP negative) positioned mostly in places where they can participate in clearance function e. g. submucosal fibroblasts in the small intestine and fibroblasts surrounding the pulmonary arteries. Interestingly most of these locations e.g urinary bladder are also going through huge elastic movements that may require a certain type of expression profile on the fibroblasts.

Disturbances in the fibroblast function have been suggested to lead to chronic inflammation and ectopic accumulation of lymphoid cells in structures called tertiary lymphoid organs (Buckley et al., 2001; Drayton et al., 2006; Roozendaal and Mebius, 2011). Such findings are often associated with autoimmune diseases such as Rheumatoid arthritis, Sjögrens's syndrome and multiple sclerosis and resemble closely the lymphoid cell infiltrates seen in multiple organs of SCARA5 deficient mice (Drayton et al., 2006). The additional finding of antinuclear antibodies in the mice further suggests that the lack of SCARA5 in the fibroblasts leads to autoimmune disease and dysfunctional regulation of adaptive immunity. It is thought that autoantibodies arise from dysregulation of T- and B-cell differentiation, that are both dependent on the cytokine environment and interaction with antigen presenting dendritic cells (Buckley et al., 2001; Drayton et al., 2006). Interestingly, it was shown that thymic fibroblasts play an important role in T-cell development and a PDGFR α - positive fibroblast population, which we could also identify as SCARA5 positive, is involved (Gray et al., 2007; Suniara et al., 2000). Furthermore, both SR-A and MARCO have been associated with autoimmune disease SLE. The patients develop autoantibodies against the receptors that lead to impaired removal of apoptotic cells (Chen et al., 2011; Rogers et al., 2009; Wermeling et al., 2007). How these receptors function in regulating autoimmunity is not fully understood but they may well share the mechanisms with SCARA5. Some studies how the processes works in case of MARCO and SR-A have been made. For example, MARCO positive cells have been shown to interact with B-cells (Karlsson et al., 2003). SR-A was shown to shape antigen presentation by mediating antigen transfer between B-cells and macrophages (Harvey et al., 2008; Raycroft et al., 2012). SR-A and T-cell involvement has also been studied in hepatitis patients and EAE multiple sclerosis model (Levy-Barazany and Frenkel, 2012; Zuo et al., 2013).

Another interesting aspect of fibroblast function and SCARA5's role in it is cancer-associated fibroblasts (CAF). CAFs are important regulators of the tumor progression, growth, and spreading (Kalluri and Zeisberg, 2006). As discussed earlier, fibroblasts are a heterogenous population and in tumor environment fibroblasts with varying degrees of PDGFR α , ASMA and FSP expression are found (Anderberg and Pietras, 2009; Kalluri and Zeisberg, 2006). The ASMA and FSP expressing fibroblasts are regarded as activated fibroblasts gradually losing the expression of PDGFR α (Anderberg et al., 2009; Kalluri and Zeisberg, 2006). Do SCARA5 positive fibroblasts go through this process since they are positive for PDGFR α but negative for FSP? Interestingly, SCARA5 expression was downregulated in several human hepatocellular carcinoma cell lines and tumor samples. It was also shown to limit the tumor growth and metastasis in mice (Huang et al., 2010; Yan et al., 2012).

6 CONCLUDING REMARKS

This study clearly demonstrates that scavenger receptors MARCO and SCARA5 play a role in host defense function - whether against endogenous proteins or microorganisms. Importantly, these two receptors are located on very different cell types, macrophages and fibroblasts, respectively. However, even after we were able to identify the SRCR domain as the main functional domain of MARCO and analyze the structure in detail, it is still not totally clear how the binding of large ligands such as bacteria or modified LDL is truly achieved. Everything suggests that multiple copies of the domain are required. Indeed there are three SRCR domains in each naturally occurring trimeric form of cell surface expressed MARCO, is this really enough? The dimensions still suggest otherwise and maybe oligomerization is needed as was suggested by the finding of the dimeric structure and oligomers seen in the rotary shadowing images (Sankala et al., 2002). Keeping this in mind, it would be interesting to solve the structure of a trimeric scavenger receptor molecule with a bound ligand. Some trials were naturally made, but the soluble form of MARCO is quite large molecule to work with and poses problems not only in the production, but also in the solubility and heterogeneity of the protein – problems not very desirable for any crystallization trials. There are things to be done to circumvent all these problems, but maybe the most natural choice is to look at the structure with the help of single particle microscopy and electron tomography.

We have also seen that MARCO deficient mice are more susceptible to various infections and therefore it raises a question whether these proteins could be used as therapeutic agents. This would be a quite attractive alternative to the use of antibiotics. These proteins sequences have been evolutionally tested for thousands of years and therefore bacteria are unlikely able to generate resistance against these proteins. It seems also natural to use our own innate immune system to boost the immune reactions, since these molecules are unlikely to have as dramatic toxic effects as some pharmaceuticals. Here, however it is good to keep in mind that both MARCO and SCARA5 seem to be involved in the development of autoimmune reactions and the question is whether a treatment with soluble receptors would lead to the development of an autoimmune disease or could it actually be the solution resolving one. Regardless, it is clear that more studies need to be made in order to understand the mechanism behind their function.

When it comes to SCARA5 and the knockout mice generated in this study, I feel that we have basically just now generated the tools to study its function and function of fibroblasts, the most common cell type of the interstitial connective tissue. The mice studied in this study were unchallenged, so what happens to the mice under different autoimmune disease models? What happens if they are infected with different bacteria? What is the role of these fibroblasts at different sites? Are the mice more prone to develop tumors? What is the role of SCARA5 in the testis – why do the male mice become infertile? I am looking forward to finding answers to these and countless other questions that are bound to arise.

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