Developmental Role and Ligand Binding Properties of Macrophage Scavenger Receptor MARCO

Yunying Chen

Stockholm 2006
TO MY FAMILY
Macrophages express several cell surface receptors, that mediate the recognition of a wide range of endogenous and exogenous ligands. In this manner, macrophages are able to perform their multiple biological functions in regulation of homeostasis and host defense. These surface receptors recognize ligands, either indirectly by an opsonization mechanism, or directly by pattern recognition.

Pattern recognition receptors (PRR) are important in the initiation of innate immune response. Scavenger receptors (SRs) are PRRs with a common specificity of binding to polyanionic ligands. MARCO (MACrophage Receptor with a COllagenous structure) and SR-A (Scavenger Receptor A), that belong to class A SRs, share domain structural properties and ligand repertoire, but they differ with respect to tissue distribution and ligand binding properties.

In contrast to the broad expression pattern of SR-A, expression of MARCO is restricted to a subset of tissue macrophages, i.e. macrophages in the spleen marginal zone (MZ), lymph nodes, and resident macrophages of the peritoneal cavity. The strategic positioning of MARCO on the spleen MZ macrophages suggests a role of MARCO in the removal of circulating organisms, as well as in the initiation of TI-2 (Thymus-Independent type 2) response, which depends on an intact spleen MZ. To study the in vivo biological role of MARCO, we have generated MARCO-deficient and MARCO/SR-A-double-deficient mice. These mice appear normal in a germ-free environment. However, the development of the spleen MZ is defective in these mice. Knockout mice showed delayed development of the MZ during ontogeny, and its microarchitecture was still immature at adult age. During ontogeny, MARCO-positive cells are spread throughout the entire spleen at the day of birth, but they form the MZ structure in the following week. Following the formation of the MARCO-positive MZ, the macrophages started to develop and differentiate to express other receptors, such as SIGNR1 and Siglec-1. A similar finding was observed during reappearance of spleen macrophages after liposome-induced spleen injury in adult mice. The defective microarchitecture of the spleen MZ led to an impaired TI-2 response in knockout mice, which was observed when mice were challenged with a pneumococcal
polysaccharide vaccine, a TI-2 antigen. Besides the defects in the spleen MZ, the ablation of MARCO results in a significant reduction in the size of resident peritoneal macrophage population. Since SR-A is also strongly expressed on macrophages in the spleen MZ and peritoneal cavity, all the above-described phenotypes were, not surprisingly, more striking in mice lacking both MARCO and SR-A receptors. These findings suggest that MARCO and SR-A, in addition to being bacteria-binding receptors, possibly interact with endogenous ligands through which they regulate the positioning and differentiation of macrophages in vivo.

Identification of ligands is an important aspect of PRR research. We have taken the advantage of an unbiased approach, phage display, to search for novel MARCO ligands using soluble recombinant MARCO (sMARCO) protein. This resulted in the enrichment of several hydrophobic peptides, which was contrary to the previous understanding that the ligands of scavenger receptors are polyanionic. BIAcore analysis confirmed that the hydrophobic peptides are ligands for sMARCO, and have a higher affinity than the known MARCO ligands LPS and LTA. Database search suggested that the most enriched peptide sequence represents complement C4, but so far we have not got convincing experimental results to support this suggestion. Further work with the two most enriched peptides demonstrated that these peptides bind to the SRCR domain of MARCO molecule. A study with chimeric scavenger receptors indicated that even minor sequence changes in the SRCR domain can have profound effects on the binding of the prototypic scavenger receptor ligand, AcLDL. This study strengthens the notion that the SRCR domain is the major ligand-binding domain in MARCO, and that this domain contains multiple ligand-binding sites.

The results of the last part of my thesis work demonstrate that MARCO recognizes Neisseria meningitidis (NM), an important human pathogen. Interestingly, we found that MARCO binds both wild-type NM as well as a mutant strain which lacks lipid A, indicating that LPS and LTA are not the only bacterial ligands of MARCO. However, although the studies with the peritoneal macrophages from different knockout strains indicate that both MARCO and SR-A participate in the binding/phagocytosis of NM, neither receptor is required for NM-stimulated TNF-α and nitric oxide production. Thus, the results suggest that TLR-dependent induction of MARCO during infection plays primarily a role in the clearance of invading pathogens.
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Yunying Chen
Stockholm, July 2006
ORIGINAL PUBLICATIONS


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<table>
<thead>
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<th>Definition</th>
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<tbody>
<tr>
<td>AcLDL</td>
<td>Acetylated low-density lipoprotein</td>
</tr>
<tr>
<td>AM</td>
<td>Alveolar macrophage</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTA</td>
<td>Lipoteichoic acid</td>
</tr>
<tr>
<td>MAdCAM-1</td>
<td>Mucosal addressin cellular adhesion molecule-1</td>
</tr>
<tr>
<td>MARCO</td>
<td>Macrophage receptor with a collagenous structure</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Monocyte-colony stimulating factor</td>
</tr>
<tr>
<td>MMM</td>
<td>Marginal zone metallophilic macrophage</td>
</tr>
<tr>
<td>MZ</td>
<td>Marginal zone</td>
</tr>
<tr>
<td>MZM</td>
<td>Marginal zone macrophage</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>OxLDL</td>
<td>Oxidized low-density lipoprotein</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern-recognition receptor</td>
</tr>
<tr>
<td>Siglec-1</td>
<td>Sialic acid-binding Ig-like lectin-1</td>
</tr>
<tr>
<td>SIGNR1</td>
<td>Specific intracellular adhesion molecule-grabbing nonintegrin receptor 1</td>
</tr>
<tr>
<td>SR-A</td>
<td>Scavenger receptor A</td>
</tr>
<tr>
<td>SRCR</td>
<td>Scavenger receptor cysteine rich domain</td>
</tr>
<tr>
<td>TD</td>
<td>Thymus-dependent</td>
</tr>
<tr>
<td>TI-2</td>
<td>Thymus-independent type 2</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
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**AIMS OF THE PRESENT STUDY**

**MATERIALS AND METHODS**

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REVIEW OF LITERATURE

1. Tissue Homeostasis and Host Defense, the Tasks of the Immune System

To keep clean from internal debris, dying and malignant cells; to repair after wounding and inflammation; to protect against aggressions from external sources, animals have evolved a wide variety of defense mechanisms that are operated by the immune system.

The immune system in vertebrates is divided into innate and adaptive immune systems. The adaptive system is the result of evolution in vertebrates only, while all lower species (99% of animals in total) survive well with only innate immunity for the protection. Also, in most cases, vertebrates do perfectly well by only using the innate immune system that provides protection against infections and maintains tissue homeostasis without tissue damage. However, because vertebrates are complex organisms, the adaptive immune system has evolved to provide them with immunological memory, and to protect them from the more complex diseases, such as cancer or virus infections. This review will describe selective players in innate immunity, at the organ (spleen), cellular (macrophages), and molecular (pattern-recognition receptors) levels.

2. Innate Immune System

The innate immune system does not only function as the first line of host defense, but also as an activator and controller of the adaptive immune response. Along with these functions, the components of the innate immune system play a broader role in tissue homeostasis. Also, it has become recognized that innate immunity is ancient in
evolution. All these factors have led to increased interest in this form of immune system.

In contrast to adaptive immunity, the characteristics of innate immunity are ascribed to the different characteristics of receptors involved. These are summarized in Table 1 (the receptors of the innate system will be reviewed in Chapter 6 in more detail). The innate immune system relies on receptors that are germline-encoded, whereas the adaptive system uses antigen receptors encoded by genes through somatic gene rearrangement. As a result, receptors of the innate system are deployed nonclonally, whereas the antigen receptors of the adaptive immune system are clonally distributed on individual lymphocytes (Janeway et al. 2001).

<table>
<thead>
<tr>
<th>Receptor characteristic</th>
<th>Innate immunity</th>
<th>Adaptive immunity</th>
</tr>
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<tbody>
<tr>
<td>Specific inherited in the gene</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Expressed by all cells of a particular type (e.g. macrophages)</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Trigger immediate response</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Recognize broad classes of pathogens</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Encoded in multiple gene segments</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Require gene rearrangement</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Clonal distribution</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Able to recognize a wide variety of molecular structures</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 1. Comparison of the characteristics of receptors of the innate and adaptive immune systems. Adapted from Janeway et al. 2001.

Besides the physical barriers of epithelia, the components of the innate immune system are complement proteins (the activated complement proteins can destroy invaders directly, or enhance the function of phagocytic cells by opsonization and recruit other immune cells to the site of inflammation), professional phagocytes (usually they are macrophages, neutrophils, and immature dendritic cells (DCs)), and natural killer cells (triggered by missing self and regulated by competing inhibitory and activating receptors). The players of the adaptive system are B and T lymphocytes, which have to be activated by antigen-presenting cells (APCs). APCs, on the other hand, usually can be the phagocytic cells of the innate system.
3. Macrophages

3.1. Macrophages, the “Big Eaters”

The immune system operates the body’s defensive mechanisms for tissue homeostasis and host defense. One of the defenders that resides in all tissues of the body is the famous player, the macrophage. This player received its name because of its capability to function as a professional phagocyte. “Macro” means large in Greek, and the macrophage is a large cell indeed. The ending “phage” also comes from a Greek word, meaning “to eat”. In fact, macrophages work as garbage collectors in normal tissues, and are also fond of eating invaders. All tissues contain macrophages in a normal steady-state situation, but their numbers increase significantly during infection, inflammation, wounding, or malignancy. In this way, macrophages play an important role both in tissue homeostasis and host defense.

3.2. Where do macrophages come from?

The myeloid progenitor in the bone marrow is the common origin of blood monocytes, immature DCs and granulocytes, such as neutrophils. After circulating in the peripheral blood for several days, monocytes and immature DCs enter tissues, and monocytes give rise to a variety of tissue macrophages. Neutrophils circulate in the blood and enter tissues only when recruited to sites of inflammation or infection. Macrophages, immature DCs and neutrophils are professional phagocytes, but neutrophils differ from macrophages and DCs in being able only to perform phagocytosis, not antigen presentation. Besides being replenished by blood monocytes, many of tissue macrophages can also be derived from local proliferation.

3.3. Macrophages play a role in host defense, as well as in normal tissue homeostasis.

It is well known that macrophages are not only important players in the innate immune system, but they also crucially influence and induce the adaptive immune responses. In the innate system, macrophages are able to eliminate pathogens directly without help of the adaptive immune system by their phagocytic and destructive capacity. Macrophages also produce cytokines and chemokines, resulting in the
recruitment of other immune cells to the site of infection. These cytokines can also influence the Th1/Th2 balance, thus deciding the direction of the adaptive immune responses. Furthermore, macrophages are APCs, i.e. they present antigens to T lymphocytes and activate the adaptive immune response directly.

Macrophages are multi-functional cells, with specialized biological roles in different anatomical locations. For example, macrophages in bone, the osteoclasts, are important for bone remodeling, allowing bone to remain strong and flexible. They also keep the serum concentration of calcium and phosphate under tight control (Quinn & Gillespie 2005). Macrophages in skin participate in the process of wound healing, remove debris from wounds, release growth factors, and reorganize the extracellular matrix (Diegelmann & Evans 2004). Thymic macrophages ingest apoptotic T cells during their development in thymus (Surh & Sprent 1994). Tingible body macrophages, found in germinal centers, phagocytose apoptotic B cells generated during the development of an adaptive immune response (Tabe et al. 1996). Microglia, the macrophages of the central nerve system, spring into action when damage occurs, creating a protective barrier around the injury and cleaning up dead cells and other debris (Kadiu et al. 2005). Macrophages in the gut lamina propria are characterized by high phagocytic and bactericidal activity, but weak pro-inflammatory reaction (Smith et al. 2005). The alveolar macrophages in lung are involved in clearing of air-borne microorganisms and inorganic pollution particles. Kupffer cells in the liver and splenic red-pulp macrophages are important components of the body’s phagocyte system to keep the circulation clean from debris, dying cells, as well as from microorganisms (Mebius & Kraal 2005).

In short, macrophages are widely dispersed throughout whole body, where they recognize and response to both endogenous and exogenous materials, and link the innate and adaptive immune responses. In this manner, macrophages are important for the maintenance of tissue homeostasis, not only in such normal processes as tissue remodeling and repair, but also in host defense against invading pathogens. The multi-functionality of macrophages relies on a limited number of receptors, which will be summarized in Chapter 6.
3. 4. Macrophages, a Heterogeneous Cell Population

The specialized function of macrophages in different organs reflects the heterogeneity of this cell type. Tissue microenvironment can markedly influence the phenotype of tissue resident macrophages, which has been uncovered through studies with monoclonal antibodies (Dijkstra et al. 1985; Kraal & Janse 1986). In addition to the heterogeneity of macrophages observed in different organs, in a single organ, macrophages exhibit different phenotypes depending on special microenvironments. The rodent spleen is a particularly good example for this.

4. Spleen

The spleen is the largest peripheral lymphoid organ, as well as the body’s largest filter of the blood. Its unique tissue structure, together with the enrichment of heterogeneous macrophages and lymphocytes enables the spleen to remove aging erythrocytes, blood-borne microorganisms and cellular debris, as well as to induce adaptive immunity.

4. 1. Structure of the Spleen

The spleen can be divided microscopically into three compartments, the red pulp, the white pulp and the marginal zone (MZ). The *red pulp* is composed of the venous sinusoidal system, named after the large, blood-filled sinuses, which constitute an important blood-filtration system. The adaptive immune responses are induced in the *white pulp*, which is organized into T- and B-cell compartments in a very similar way as the lymph node is organized. The *MZ* is the compartment between the red and white pulp, well equipped with MZ macrophages, DCs and MZ B cells (Kraal 1992; Mebius & Kraal 2005). This region is not only important for the first line of defense against blood-borne antigens, but it is also unique in its ability to rapidly initiate the anti-TI-2 (Thymus-independent type 2) responses (Zandvoort & Timens 2002).

The complexity of the spleen structure is related to the complexity of its vascular system. The spleen *afferent artery* branches into *central arteries*, which then branch into smaller arterioles. These small branches form the capillary meshwork and
are sheathed by lymphoid tissues, which form the white pulp. Some smaller arterioles traverse the white pulp into the red pulp, from where the blood runs into *venous sinuses*, which drain into an *afferent splenic vein*. The venous sinuses are not lined by endothelium, but by cytoplasmic processes of reticular cells. Due to this open circulation, the blood comes into contact with the numerous red-pulp macrophages and is effectively filtered. Some other smaller arterioles terminate and open in the *MZ sinus* between the white pulp and the MZ. The blood flow slows down in the MZ sinus, which gives an opportunity for the MZ cells to react with incoming cells and antigens. The incoming cells, on the other hand, can utilize this route to migrate into the white pulp, or they can stay in the MZ itself. In turn, the cells in the MZ and the white pulp can enter the bloodstream via the sinus. In the human spleen, the presence of the MZ sinus has not been demonstrated. The MZ is divided into an inner and an outer compartment by a special type of fibroblast. An additional compartment, termed the *perifollicular zone*, is present between the outer MZ and the red pulp. Part of the bloodstream ends in the perifollicular zone, and the endings of these capillaries are sheathed by Siglec-1 (sialic-acid-binding Ig-like lectin 1; also known as sialoadhesin) -positive macrophages (Steiniger *et al.* 1997; Mebius & Kraal 2005).

### 4.2. Heterogeneity of Splenic Macrophages

The spleen is rich in subpopulations of macrophages, which differ in receptor expression, micro-anatomical location, life history and function.

#### 4.2.1. Red-Pulp Macrophages

By localizing in the open venous sinusoidal system, the red-pulp macrophages are ideally positioned to perform their blood-filtration function. Indeed, these cells are well equipped for fighting against bacteria and facilitating iron metabolism by expression of several receptors, such as *CD163*, a hemoglobin-specific macrophage surface receptor scavenging hemoglobin from the circulation (Kristiansen *et al.* 2001), and *NRAMP1* (Natural-Resistance-Associated Macrophage Protein 1), an integral membrane protein expressed in the lysosomal compartment of macrophages, which is recruited to the membrane of bacterial phagosomes where it affects intracellular microbial replication (Gruenheid & Gros 2000).
4. 2. 2. White-Pulp Macrophages

Tingible body macrophages are localized in the white pulp. They express and secrete high levels of *MFG-E8* (Milk Fat Globule-EGF factor 8), a glycoprotein that binds to apoptotic B cells in the germinal centers by recognizing phosphatidylserine. A study with the MFG-E8-deficient mice showed that lack of MFG-E8 impairs the ability of tingible body macrophages to engulf and remove apoptotic cells, which leads to the development of a lupus-like autoimmune disease (Hanayama et al. 2006). MFG-E8 is not expressed by the thymic macrophages and is thus very likely not involved in the clearance of apoptotic thymocytes (Hanayama et al. 2004). Tingible body macrophages are negative for the expression of *F4/80*, which is expressed by most tissue macrophage populations.

4. 2. 3. Macrophages and MZ B cells in the Spleen Marginal Zone

Cells in the MZ are constantly exposed to blood and antigens that have an access to the systemic circulation. The cells in the MZ are macrophages, DCs, MZ B cells and reticular fibroblasts (*Figure 1*). Reticular fibroblasts form the framework of the MZ in which the other cell types then distribute.

The MZ contains two subpopulations of macrophages lacking F4/80 expression. *Marginal zone metallophilic macrophages (MMMs)* are distinguishable by *Siglec-1* expression, which can be identified by the *MOMA-1* antibody (Kraal & Janse 1986; Munday et al. 1999). They form the inner ring of the MZ adjacent to the white pulp and the MZ sinus (*Figure 1*). A study with M-CSF deficient mice showed that these mice lack the MMMs but maintain other splenic macrophages, which indicates heterogeneity in the development, differentiation and maturation of the splenic macrophages (Witmer-Pack et al. 1993; Takahashi et al. 1994). The function of the MMMs has not been well addressed. They may participate in host defense against viral infections (Eloranta & Alm 1999). An *in vitro* study showed that Siglec-1 mediates the capture and uptake of lipopolysaccharide (LPS) from *Neisseria meningitides* (Jones et al. 2003).
Marginal zone macrophages (MZMs) form the outer ring of the MZ (Figure 1). This strategic positioning indicates a role for this population in the first line of host defense against blood-borne pathogens. The MZMs express a range of pattern-recognition receptors (PRRs), such as Toll-like receptors, SR-A (scavenger receptor A), and Dectin-2, which play important roles in the innate immune responses against microbial infections. Besides these common PRRs, the MZMs express two PRRs that are unique to this region. One of these, SIGNR1, a C-type lectin recognized by the ERTR-9 antibody (Dijkstra et al. 1985), binds polysaccharide antigens on Streptococcus pneumoniae and Mycobacterium tuberculosis (Kang et al. 2004; Koppel et al. 2004). SIGNR1 is also found to bind viruses (Marzi et al. 2004) and yeasts such as C. albicans (Taylor et al. 2004). MARCO (MAcrophage Receptor with a COllagenous domain), a class A scavenger receptor, is also a bacteria-binding receptor (Elomaa et al. 1995) that contributes to the capture and clearance of circulating microorganisms in the MZ. MARCO expression by the MZMs is also important for the retention of the MZ B cells (Karlsson et al. 2003). In turn, there is evidence indicating that B cells are crucial for both development and maintenance of the MZ. Indeed, the spleens of B cell-deficient or -depleted mice were found to lack both MMMs and MZMs, as well as MAdCAM-1 (Mucosal Addressin Cellular Adhesion Molecule-1) -positive MZ sinus-lining cells (Figure 1) (Dingjan et al. 1998;
Nolte et al. 2004). These findings demonstrate the importance of cellular interactions for the integrity of the MZ.

MZ B cells are one of the first cell populations encountering invading blood-borne pathogens. They are responsible for rapid antibody responses after antigen stimulation, particularly for the responses against blood-borne TI-2 antigens (Kraal 1992; Guinamard et al. 2000) (see the next chapter). The MZ B cells have a distinct phenotype compared with the more common follicular B cells. The MZ B cells express high levels of IgM, CD1d, CD9, and CD21, but low levels of IgD, CD23 and B220. The follicular B cells express high levels of IgD and CD23, and low levels of IgM and CD21. They do not express CD1d and CD9 (Martin & Kearney 2002).

4.2.4. Thymus-Independent (TI) Immune Response

Based on immunogenicity in congenitally athymic (nu/nu) mice and mice with the X-linked immune B cell defect (xid-mice), antigens are divided into thymus-dependent (TD), and thymus-independent type 1 (TI-1) and type 2 (TI-2) antigens. TD antigens fail to induce a response in nu/nu mice. TI-1 antigens can induce an antibody response in both nu/nu and xid-mice. TI-2 antigens induce an antibody response in nu/nu mice but fail to induce in xid-mice. Neutral polysaccharides, including capsular polysaccharide antigens of encapsulated bacteria, are typical TI-2 antigens. The MZ B cells and B1 B cells in the peritoneal cavity are the major cells responsible for the TI immune responses (Martin et al. 2001). Although these cells do not need “help” from T cells to generate a TI immune response, they are influenced by macrophage-like accessory cells and the presence of T cells for the proper responses (Garg et al. 1996; Janeway et al. 2001; Balazs et al. 2002).

4.3. Immunological Role of the Spleen

In the spleen, the innate immune response against blood-borne pathogens relies mainly on the MZ macrophages and the MZ B cells, as summarized above. The marginal dendritic cells, activated by the incoming pathogens in the MZ, are able to migrate into the white pulp to initiate the adaptive immune responses. Similarly, activated circulating dendritic cells can migrate into the spleen white pulp through the MZ.
The proper development of the spleen MZ is delayed until about the age of two years in human and until 3-4 weeks after birth in rodents. The maturation of the MZ macrophages and the MZ B cells is delayed during early life. The precise cellular and molecular basis for this delay remains unclear, but it correlates with the increased susceptibility of the infants below the age of two years to infections with such encapsulated bacteria as *Streptococcus pneumoniae*, *Neisseria meningitides* or *Haemophilus influenzae* (Kruschinski et al. 2004). The importance of the spleen in the protection against bacterial infections has also been observed in studies of splenectomized patients and mice who are unable to mount effective responses to several bacterial products. The splenectomy in human therefore leads to the lifelong requirement for prophylactic intake of antibiotics (Amlot & Hayes 1985).

5. Lymph Nodes

The spleen collects antigens from the blood, whereas the antigens from the peripheral sites of infection are collected by draining lymph nodes, and the antigens from the epithelial surfaces of the gastrointestinal tract or respiratory epithelium are collected by mucosal associated lymphoid tissues. Although very different in appearance, the lymph nodes, spleen, and mucosal associated lymphoid tissues all share the same basic architecture. They are the peripheral lymphoid organs specialized to trap antigens, to allow the initiation of adaptive immune responses, and to provide signals that sustain recirculating lymphocytes.

The lymph nodes are located at the points of convergence of vessels of the lymphatic system that collects extracellular fluid (that is called lymph) from the tissues. The fluid is filtered in the lymph nodes, after which it returns to the blood. A lymph node consists of an outermost cortex and an inner medulla. The cortex is composed of an outer cortex of B cells organized into follicles, and paracortical area made up of T cells and dendritic cells. The medulla consists of strings of macrophages and antibody-secreting plasma cells. The macrophages in the medulla are important for cleaning lymph by phagocytosis.
6. Pattern-Recognition Receptors (PRRs)

The professional phagocytic cells of the innate immune system recognize ligands either indirectly through opsonization (antibodies and complement opsonize pathogens or particles for destruction by phagocytic cells through Fc- or complement receptors) or directly by pattern-recognition receptors (PRRs) through the recognition of different conserved molecular patterns. In contrast to lymphocyte receptors of the adaptive immune system, PRRs are germline-encoded, nonclonal, expressed on all cells of a given type, and independent of immunological memory (Table 1).

6. 1. Pattern recognition involves both host defense and tissue homeostasis.

The original concept of pattern recognition, proposed by Janeway and Medzhitov (Janeway & Medzhitov 2002), emphasizes host-microbial interactions, and is based on the recognition of microbial nonself structures, so-called pathogen-associated molecular patterns (PAMPs), by a limited number of germ-line encoded PRRs. However, it has become clear that the concept of pattern recognition needs to be broadened, because PRRs recognize endogenous ligands in host as well. These receptors, therefore, play a dual role in tissue homeostasis and host defense (Gordon 2002).

The molecular range of ligands of PRRs is very wide, including proteins, lipids, carbohydrates and nucleic acids of both exogenous and endogenous sources. A single PRR is often able to recognize multiple ligands through relatively weak interactions. Some ligands, such as LPS, can bind to several distinct PRRs. Furthermore, different PRRs can cooperate as a receptor complex for certain ligands, e.g. CD14, TLR4 and MD2 form the receptor complex for LPS binding.

6. 2. PRR Categories

According to the functional properties, PRRs can be simply divided into endocytic PRRs and signaling PRRs. Endocytic PRRs, such as scavenger receptors, mannose receptors and β-glucan receptors, promote the attachment, engulfment and destruction of microorganisms by phagocytes. Signaling PRRs, such as Toll-like receptors (TLRs) and NOD-LRR proteins, act as pathogen sensors by triggering intracellular signaling
and orchestrating the inflammatory responses. In the following paragraphs, several representative PRRs will be described briefly.

6. 2. 1. Toll-Like Receptors (TLRs)

One of the hallmarks of the innate immune research has been the discovery of Toll-like receptors (TLRs), because research on them has provided insight into the signaling mechanisms in anti-microbial host defense.

TLRs are named after the *Drosophila Toll* receptor. This *Drosophila* protein was originally identified as a protein important for controlling dorsoventral polarization during embryogenesis. Only later it was shown to have a role in anti-fungal host defense (Lemaitre et al. 1996). The first mammalian Toll homologue was cloned 1997 (now called TLR4). The *Drosophila* Toll and the mammalian TLRs are structurally similar, and both signal through the NF-κB pathway (Medzhitov et al. 1997). In *Drosophila*, the Toll family consists of 9 members, of which most are not involved in anti-microbial host defense. In mammals, 12 members of the TLR family have been identified. The members of the family sense different microbial components, as summarized in Table 2 (Akira et al. 2006).

TLRs are type I transmembrane proteins with an extracellular domain composed of leucine-rich repeats (LRR) mediating ligand binding, and a cytoplasmic signaling domain homologous to the cytoplasmic domain of the human interleukin (IL)-1 receptor, termed as *Toll/IL-1R homologue (TIR) domain* (Bowie & O’Neill 2000). The engagement of TLRs with different microbial components triggers the activation of signaling cascades, leading to the activation of genes involved in the anti-microbial host defense. These processes are mediated by *TIR-domain-containing adaptor proteins*, whose association with the TIR domains of TLRs is induced by ligand binding. Four TLR-interacting adaptors, MyD88, TIRAP /Mal (Fitzgerald et al. 2001; Yamamoto et al. 2002), TRIF (or TICAM1) (Hoebe et al. 2003; Yamamoto et al. 2003a), and TRAM (Yamamoto et al. 2003b), have been identified. Depending on the usage of the adaptors, TLRs can active MyD88-dependent and -independent signaling pathways, leading to the production of proinflammatory cytokines and type I interferons, respectively. In this manner, distinct TLR ligands can induce different immune responses.
### Microbial components

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Species</th>
<th>TLR Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>Gram-negative bacteria</td>
<td>TLR4</td>
</tr>
<tr>
<td>Diacyl lipopeptides</td>
<td>Mycoplasma</td>
<td>TLR6/TLR2</td>
</tr>
<tr>
<td>Tricacyl lipopeptides</td>
<td>Bacteria and mycobacteria</td>
<td>TLR1/TLR2</td>
</tr>
<tr>
<td>LTA</td>
<td>Group B <em>Streptococcus</em></td>
<td>TLR6/TLR2</td>
</tr>
<tr>
<td>PG</td>
<td>Gram-positive bacteria</td>
<td>TLR2</td>
</tr>
<tr>
<td>Porins</td>
<td><em>Neisseria</em></td>
<td>TLR2</td>
</tr>
<tr>
<td>Lipoarabinomannan</td>
<td>Mycobacteria</td>
<td>TLR2</td>
</tr>
<tr>
<td>Flagellin</td>
<td>Flagellated bacteria</td>
<td>TLR5</td>
</tr>
<tr>
<td>CpG-DNA</td>
<td>Bacteria and mycobacteria</td>
<td>TLR9</td>
</tr>
<tr>
<td>ND (not determined)</td>
<td>Uropathogenic bacteria</td>
<td>TLR11</td>
</tr>
</tbody>
</table>

**Fungus**

| Zymosan                        | *Saccharomyces cerevisiae*           | TLR6/TLR2 |
| Phospholipomannan              | *Candida albicans*                  | TLR2      |
| Mannan                         | *Candida albicans*                  | TLR4      |
| Glucuronoxylomannan            | *Cryptococcus neoformans*           | TLR2 and 4 |

**Parasites**

| tGPI-mutin                     | *Trypanosoma*                       | TLR2      |
| Glycoinositolphospholipids     | *Trypanosoma*                       | TLR4      |
| Hemozoin                      | *Plasmodium*                        | TLR9      |
| Profilin-like molecule         | *Toxoplasma gondii*                 | TLR11     |

**Viruses**

| DNA                            | Viruses                             | TLR9      |
| dsRNA                          | Viruses                             | TLR3      |
| ssRNA                          | RNA viruses                         | TLR7 and 8|
| Envelope proteins              | RSV, MMTV                           | TLR4      |
| Hemagglutinin protein          | Measles virus                       | TLR2      |
| ND (not determined)            | HCMV, HSV1                          | TLR2      |

**Host**

| Heat-shock protein 60, 70      |                                    | TLR4      |
| Fibrinogen                    |                                    | TLR4      |

**Table 2. TLR recognition of microbial components.** Adapted from Akira et al. 2006.

TLRs are expressed on various immune cells, including macrophages, DCs, B cells, specific type of T cells, as well as on fibroblasts and epithelial cells. Some TLRs, such as TLRs 1, 2, 4, 5, and 6, that sense microbial cell wall components, are expressed on the cell surface, whereas some others are expressed in intracellular organelles, endosomes or lysosomes. These include TLRs 3, 7, 8, and 9. Consequently, the ligands of these TLRs, mainly nucleic acids, require internalization.
to the endosomal compartment before signaling can occur. On the other hand, TLRs cannot sense pathogens that have invaded the cytosol. These are detected by cytoplasmic PRRs, such as the NOD-LRR and CARD-helicase proteins, which are implicated in the sensing of intracellular bacterial and viral components, respectively (Akira et al. 2006).

TLRs are not only crucial for innate immunity, but also important for the adaptive immune response. It is now well known that the TLRs expressed on DCs induce DC maturation required for the activation and differentiation of T helper cells, which then activate B cells for antibody responses (Pasare & Medzhitov 2005b). Furthermore, a recent study with mice lacking the adaptor protein MyD88 indicates that the TLRs expressed on B cells can activate these cells directly. Although these mice have defective T cell activation and impaired antibody responses to TD antigens, the restoration of the T cell defects did not restore the B cell responses, indicating that the TLRs expressed by B cells have a direct role in the antibody responses. Further work led to the conclusion that TLR signaling affects multiple stages of B cell activation and is required for optimal antibody responses to TD antigens (Pasare & Medzhitov 2005a). Similar conclusions have been obtained also in a study with human B cells (Ruprecht & Lanzavecchia 2006). Such direct activation of B cells by TLR signaling suggests that the inappropriate activation of self-reactive B cells by their own TLRs can induce autoimmune responses.

6.2.2. NOD-LRR Proteins

NOD-LRR proteins are cytoplasmic proteins composed of three different types of domains, a C-terminal LRR domain for ligand binding, a nucleotide binding oligomerization domain (NOD domain) and an N-terminal effector binding domain (EBD domain), such as CARD, PYRIN, or BIR domain, for the initiation of signaling (Inohara et al. 2005; Martinon & Tschopp 2005).

Several members of this protein family have been shown to mediate the recognition of bacterial components in the cell cytosol. For example, NOD1 (Chamaillard et al. 2003; Girardin et al. 2003a) and NOD2 (Girardin et al. 2003b), both containing a CARD signaling domain, recognize naturally occurring bacterial peptidoglycan fragments, and active the NF-κB signaling pathway. NALP3, belonging to the NALP subfamily with a PYRIN signaling domain, is involved in the
recognition of bacterial RNA (Kanneganti et al. 2006), ATP (Mariathasan et al. 2006), and uric-acid crystals (Martinon et al. 2006). Ligand recognition leads to the activation of the caspase-1 signaling pathway and cleavage of pro-IL-1β to mature IL-1β. IPAF, another CARD containing NOD-PRR protein, recognizes *Salmonella typhimurium*, and induces caspase-1 activation (Mariathasan et al. 2004). NAIP5, a NOD-PRR protein containing a BIR signaling domain, is associated with host susceptibility to the intracellular pathogen *Legionella pneumophila* (Diez et al. 2003).

6.2.3. Mannose Receptor (MR)

Mannose receptor (MR) is an endocytic receptor expressed by macrophages, DCs, hepatic endothelial cells, and on some other cell types (Stahl & Ezekowitz 1998). It is a type I C-type lectin receptor with a long extracellular portion including a N-terminal cysteine-rich domain, a fibronectin type II (FNII) domain and a unique series of eight C-type lectin-like domains, the carbohydrate–recognition domains (CRDs), which endow the receptor with the capability to recognize mannosyl-, fucosyl- or N-acetylglucosamidyl-terminated glycoconjugates (Taylor & Drickamer 1993). These types of carbohydrates are abundant on the surface of microorganisms, and indeed several in vitro studies have suggested a role for MR in host defense. However, two fungal infection studies have been reported, but in neither of them were MR-deficient mice found more susceptible than wild-type mice to the infection (Lee et al. 2003; Swain et al. 2003). In contrast, other studies with these mice have indicated that MR is required for rapid clearance of a subset of high-mannose serum glycoproteins that are normally elevated during inflammation and wound healing. Thus MR appears to act as an essential regulator of serum glycoprotein homeostasis (Lee et al. 2002).

In addition to the C-type lectin domains, MR contains another carbohydrate-binding domain, the cysteine-rich domain, which has been shown to recognize several endogenous sulfated glycoconjugates. Among the sulfated oligosaccharide-containing ligands are pituitary hormones, such as lutropin (Fiete et al. 1998; Simpson et al. 1999). One study indicates that deletion of MR affects the clearance of lutropin from circulation. Supporting this view, MR heterozygous female mice were found to have a smaller litter size due to a reduction in the rate of implantation (Mi et al. 2002).

Several studies indicate a role for MR in leukocyte trafficking. Through the cysteine-rich domain, MR can bind to the MMMs in the spleen and the B cells in
germinal centers. These interactions may help in directing MR-bearing cells toward
germinmal centers during an immune response (Martinez-Pomares et al. 1998; Leteux
et al. 2000). One study indicates that MR expressed on lymphatic endothelium may
control lymphocyte exit from lymph nodes through interaction with lymphocyte L-
selectin (Irjala et al. 2001). There is also evidence that MR binds cancer cells, and this
interaction may play a role in controlling the cancer cell traffic within the lymphatic
system (Irjala et al. 2003).

Finally, MR, as well as its relative Endo180, recognize and internalize several
types of collagens (Wienke et al. 2003; Martinez-Pomares et al. 2006). This function,
which appears to depend on receptor multimerization, is mediated by the single FNII
domain of the receptors, and may play a role in collagen turnover (East et al. 2003;
Curino et al. 2005).

6. 2. 4. Dectin-1

Dectin-1 (dendritic cell associated C-type lectin), expressed on DCs, macrophages,
monocytes, neutrophils and a subset of T cells, is a type II transmembrane protein
with an extracellular C-type lectin-like domain that recognizes β-glucans and an
endogenous undefined ligand on T cells, as well as a cytoplasmic tail with an
immunoreceptor tyrosine-based activation motif (ITAM) involved in the activation of
intracellular signaling (Brown 2006).

Both in vitro and in vivo evidence indicate that TLRs participate in the anti-
fungal host defense responses (Table 2). Several pieces of in vitro evidence suggest
that Dectin-1 plays role in anti-fungal response through recognizing β-glucans, that
constitute more than 50% of the cell walls of fungi. For example, it has been shown
that dectin-1 mediates the uptake and killing of live fungal cells, in part through
production of the respiratory burst and inflammatory cytokines and chemokines
(Steele et al. 2003; Steele et al. 2005). Several pieces of evidence also indicate that
dectin-1 and the TLR pathway cooperate in the anti-fungal responses (Brown et al.
2003; Gantner et al. 2003). In any case, the analysis of dectin-1-deficient mice is
necessary to firmly establish the role of dectin-1 in the anti-fungal host defense.

The nature of the endogenous T-cell ligand for dectin-1 is unknown, but it
might be a protein, rather than a carbohydrate, as this ligand is sensitive to trypsin
treatment, but not to a treatment with a glycosidase (Ariizumi et al. 2000). The
binding of dectin-1 with T cells increases the proliferation of T cells in vitro (Ariizumi et al. 2000). Furthermore, dectin-1 expression is detected on macrophages and DCs in the T-cell areas of the spleen, lymph nodes, and thymus (Reid et al. 2004). These data suggest a role for dectin-1 in the regulation of T cell homeostasis.

6. 2. 5. Scavenger Receptors (SRs)

Scavenger receptors (SRs) were originally identified by their ability to bind and internalize modified lipoproteins (Goldstein et al. 1979). Today, the SR superfamily is a loose group of membrane proteins expressed by macrophages, DCs, and some endothelial cell populations with the capability to bind modified low-density lipoprotein (LDL) and other polyanionic ligands. SRs are divided into 8 classes, class A to H, according to the similarity of their molecular structures (Figure 2) (Murphy et al. 2005).

![Figure 2. Schematic view of the members of the SR superfamily. Adapted from Murphy et al. 2005.](image-url)
Since the SR superfamily is too large to allow detailed description of each family member in this review, a general overview of the functions of SRs is given. The class A SR subfamily will be reviewed in more detail in chapter 7.

Many of the members of the SR superfamily recognize multiple ligands and are thus able to perform a variety of biological functions. The chemical nature of the ligands varies, but generally they have a polyanionic nature. Several SRs have been shown to be involved in host defense against bacterial, viral, parasite or fungal infection through the recognition of different microbial components. Another major class of ligands is the modified host molecules, OxLDL (oxidized LDL), β-amyloid protein, and AGE (advanced end products of glycation) -modified molecules. These molecules are products of pathophysiological processes associated with atherosclerosis, Alzheimer’s disease and diabetic complications, respectively. Although these diseases are complex, they are generally regarded as modified forms of inflammation with macrophages as major players. For example, LDL becomes oxidized and deposited in the subendothelial space of arteries when there is a too high level of the lipoprotein in the circulation. OxLDL can stimulate endothelial cells to produce chemokines and cytokines, resulting in the recruitment of macrophages to the artery wall. Macrophages take up OxLDL through SRs as the major receptors, leading to the formation of form cells, and, as the result of a positive feedback cascade, to the formation of complex atherosclerotic lesions (Itabe 2003). Similarly, AGE-modified molecules and β-amyloid protein induce macrophage recruitment to their accumulation site. SRs mediate the uptake of these materials, but the deposits can be too large to be removed, which results in frustrated phagocytosis, and induction of inflammatory responses (Yan et al. 1996; Horiuchi et al. 2005). Lastly, removal of apoptotic cells is also a very important task for SRs. In contrast to the processes described above, removal of apoptotic cells does not induce inflammatory responses. It rather induces an anti-inflammatory response, which results in the removal of apoptotic cells without any tissue damage (Platt et al. 1999). Whether and how the SRs participate in the regulation of the pro- and anti-inflammatory responses is still unclear.

Some SRs recognize also unmodified endogenous ligands (Table 3). This fact implicates a potential role for SRs in tissue homeostasis, such as in the recruitment
and retention of different immune cells in steady state, as well as in the process of inflammation (Mukhopadhyay & Gordon 2004).

<table>
<thead>
<tr>
<th>SR molecule (class)</th>
<th>Host-derived ligands</th>
<th>Modified host molecules</th>
<th>Microbe-derived ligands</th>
<th>Bacterial</th>
<th>Viral</th>
<th>Parasite</th>
<th>Fungal</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR-A (class A)</td>
<td>undefined protein in serum; gp96/GRP94; calreticulin</td>
<td>β-amyloid protein; apoptotic cells; AGE modified proteins; OxLDL; AcLDL</td>
<td>LPS; LTA; CpG-DNA; G+&amp;G-bacteria</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MARCO (class A)</td>
<td>splenic MZ B cells; UGRP-1 in lung</td>
<td>AcLDL</td>
<td>LPS; LTA G+&amp;G-bacteria</td>
<td>ND</td>
<td>ND</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>SRCL-I (class A)</td>
<td>T and Tn antigens on carcinoma cells</td>
<td>OxLDL</td>
<td>G+&amp;G-bacteria</td>
<td>ND</td>
<td>ND</td>
<td>Yeast</td>
<td></td>
</tr>
<tr>
<td>CD36 (class B)</td>
<td>thrombospondin; collagen; fatty acid; native LDL, HDL, VLDL</td>
<td>AGE; β-amyloid protein apoptotic cells; OxLDL; sickled erythrocytes</td>
<td>ND</td>
<td>ND</td>
<td>#1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>dSR-Cl (class C)</td>
<td>ND</td>
<td>AcLDL; apoptotic cells</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CD68 (class D)</td>
<td>ND</td>
<td>OxLDL</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>LOX-1 (class E)</td>
<td>fibronectin; HSP70</td>
<td>OxLDL; AGE; hypochlorite modified HDL; apoptotic cells; activated platelets</td>
<td>G+&amp;G-bacteria</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>SREC-I (class F)</td>
<td>advillin (an actin regulatory protein)</td>
<td>OxLDL; AcLDL</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>SR-POX (class G)</td>
<td>CXCR6 on subsets of T cells</td>
<td>PS; apoptotic cells; OxLDL</td>
<td>G+&amp;G-bacteria</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FEEL1/2 (class H)</td>
<td>hyaluronan receptor (FEFL2)</td>
<td>AGE; AcLDL</td>
<td>G+&amp;G-bacteria</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not determined; No, does not bind yeast; G+&G-, Gram-positive and -negative; OxLDL, Oxidized LDL; AcLDL, Acetylated LDL; PS, phosphatidylserine; #1, Plasmodium falciparum-malaria parasitized erythrocytes

Table 3. Selected ligands of the members of the SR superfamily. Summarized from reviews by Murphy et al 2005, and Mukhopadhyay & Gordon 2004.
7. Scavenger Receptors MARCO and SR-A

The class A SR subfamily is composed of MARCO and SR-A, the ‘old’ members of this subfamily, as well as the more recently identified SRCL (scavenger receptor with C-type lectin) and SCARA5 (class A scavenger receptor 5). The genes encoding SR-A and SRCL have been shown to undergo alternative splicing, and therefore, at the moment, seven structurally similar protein products can be counted as members of this subfamily.

7. 1. Class A SRs, General Information

7. 1. 1. Gene Locations of the Members of the Class A SRs

SR-A was first cloned from a bovine cDNA library (Kodama et al. 1990), after the identification of the bovine SR-A protein by exploiting the ability of macrophages to bind AcLDL (Kodama et al. 1988). MARCO was discovered and cloned in 1995 by screening a mouse macrophage library for type XIII collagen (Elomaa et al. 1995). The human and mouse SRCL were cloned in 2001 from a human placental and a mouse embryonic cDNA library, respectively (Nakamura et al. 2001a; Nakamura et al. 2001b; Ohtani et al. 2001). Recently, SCARA5 has been identified by searching a murine DNA database for sequences related to SR-A (Jiang et al. 2006). The same receptor was also identified by another group. Because of the high expression level in the testis, a name Tesr (testis expressed scavenger receptor) was suggested (Sarraj et al. 2005).

The SR-A gene, which is located on chromosome 8 in human, generates three different proteins, SR-AI, -II, and -III, by alternative splicing. The human SRCL gene is located on chromosome 18, and is alternatively spliced to generate two isoforms, SRCL-I and -II (Nakamura et al. 2001a). The human MARCO and SCARA5 genes are on chromosomes 2 and 8, respectively. In mouse, the SR-A, MARCO, SRCL and SCARA5 genes are located on chromosomes 8, 1, 18 and 14, respectively. All four genes encode transmembrane proteins with a collagenous domain suggesting that they arose from a primordial ancestral gene that underwent duplication and dispersal through the genome during evolution.
7. 1. 2. Molecular Structure of the Class A SRs

The class A SRs are homotrimeric type II transmembrane glycoproteins (Figure 2). SR-AI has a short N-terminal cytoplasmic domain, a single transmembrane domain, and a large extracellular portion comprised of a spacer, an \( \alpha \)-helical-coiled-coil domain, a collagenous domain, and a C-terminal scavenger receptor cysteine-rich (SRCR) domain (Freeman et al. 1990; Kodama et al. 1990; Doi et al. 1993). SR-AII is shorter than SR-AI as it lacks the SRCR domain (Freeman et al. 1990; Matsumoto et al. 1990). SR-AIII lacks large portions of this domain, which results in the misfolding and retention of the protein within the endoplasmic reticulum (Gough et al. 1998). MARCO is structurally similar to SR-AI except that it lacks an \( \alpha \)-helical-coiled-coil domain, but contains a much longer collagenous domain (Elomaa et al. 1995). In comparison to SR-AI, SRCL-I contains a C-terminal C-type lectin domain instead of a SRCR domain, and an additional extracellular serine/threonine-rich region. The SRCL-II isoform lacks the C-terminal lectin domain (Nakamura et al. 2001a). Jiang et al suggested that SCARA5 has five domains. Its extracellular portion is composed of a long spacer, a short collagenous domain and a SRCR domain (Jiang et al. 2006) (Table 4).

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>Full length</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR-AI</td>
<td>55</td>
<td>25</td>
<td>34</td>
<td>162</td>
<td>72</td>
<td>110</td>
<td>458</td>
</tr>
<tr>
<td>MARCO</td>
<td>49</td>
<td>25</td>
<td>75</td>
<td>No</td>
<td>270</td>
<td>99</td>
<td>518</td>
</tr>
<tr>
<td>SCARA5</td>
<td>59</td>
<td>18</td>
<td>228</td>
<td>No</td>
<td>73</td>
<td>106</td>
<td>491</td>
</tr>
<tr>
<td>SRCL-I</td>
<td>39</td>
<td>18</td>
<td>55</td>
<td>223</td>
<td>147</td>
<td>No</td>
<td>732*</td>
</tr>
</tbody>
</table>

Numbers indicate the length of the domains; No= does not contain this domain; I= N-terminal intracellular domain; II= transmembrane domain; III= spacer region; IV= \( \alpha \)-helical-coiled-coil domain; V= collagenous domain; VI= scavenger receptor cysteine-rich domain (SRCR); *SRCL has no a SRCR domain, but contains a C-type lectin domain and a serine/threonine-rich region.

Table 4. The domain composition of the murine class A SRs (references shown in the text).

7. 1. 3. Ligand Binding Properties and Expression Patterns of the Class A SRs

The collagenous domain of murine SR-A is composed of 24 uninterrupted Gly-X-Y tripeptide repeats, whereas that of murine MARCO has 89 repeats interrupted at one location (residues 174-176) by Ala-Glu-Lys. Doi et al demonstrated that for SR-A, a cluster of five lysine residues at the C-terminal end of the collagenous domain is
essential for ligand binding (Acton et al. 1993; Doi et al. 1993). MARCO contains a similar lysine-rich cluster at the corresponding position, but it does not appear to play a major role at least in the bacteria-binding function of MARCO (Brannstrom et al. 2002).

Although ligand binding to a SR most likely reflects ionic interactions, the preference of SR-A for certain nucleic acids (e.g. poly G, poly I), as well as its failure to bind polyanions such as chondroitin sulfate, suggests that both the ligand structure and charge distribution contribute to the binding specificity.

The members of the class A SR family have a similar, but not identical ligand repertoire. In vitro experiments have demonstrated that all family members bind both Gram-negative and -positive bacteria. Bacterial binding is inhibited by polyanionic macromolecules, again indicating a dependence on a charge-based recognition mechanism. Modified LDL is the prototypic ligand for SRs, but the different family members exhibit different binding characteristics for this macromolecule. SR-A binds both OxLDL and AcLDL (Suzuki et al. 1997), whereas human SRCL binds OxLDL, but not AcLDL (Ohtani et al. 2001). Mouse MARCO, but not its human counterpart, binds AcLDL (Elshourbagy et al. 2000). SCARA5 binds neither OxLDL nor AcLDL (Jiang et al. 2006).

SR-A is expressed in pathogen-free mice on most macrophage populations (Hughes et al. 1995), while the expression of MARCO is restricted to only certain populations of macrophages, such as macrophages of the spleen MZ, of lymph nodes, and of the peritoneal cavity (Elomaa et al. 1995). However, the expression of MARCO is induced on other macrophage populations in vivo and in vitro after pathogen stimulation (van der Laan et al. 1997; van der Laan et al. 1999). This induction appears to be dependent on TLRs (Doyle et al. 2004; Mukhopadhyay et al. 2004). Moreover, expression of both SR-A and MARCO has been detected also in dendritic cells (Granucci et al. 2003; Grolleau et al. 2003; Harshyne et al. 2003; Becker et al. 2006). In particular, microarray approaches have revealed that MARCO expression is strongly induced in cultured dendritic cells in response to such stimuli as bacteria, LPS, and normal or tumor tissue lysates (Granucci et al. 2003; Grolleau et al. 2003). SRCL is predominantly expressed on endothelia but not on macrophages (Ohtani et al. 2001). Similarly, SCARA5 does not appear to be expressed on macrophages, but on epithelial cells (Jiang et al. 2006).
Taken together, the ligand-binding characteristics and the expression patterns suggest partially overlapping biological functions for the members of the class A SR subfamily.

7. 2. Biological Role of MARCO and SR-A

7. 2. 1. Role of MARCO and SR-A in Tissue Homeostasis
As already mentioned above, modified LDL, the macromolecule related to the foam cell formation during atherogenesis, is the prototypic endogenous ligand for MARCO and SR-A. SR-A is expressed on foam cells, which suggests a role for this SR in atherogenesis (Matsumoto et al. 1990). In contrast, expression of MARCO in the atherosclerotic plaques has not been proven. Several mouse studies have addressed the question whether SR-A plays a role in atherogenesis. However, the results have yielded a somewhat confusing picture. Macrophages isolated from SR-A-KO (knockout) mice displayed significantly reduced uptake of modified LDL (Lougheed et al. 1997), whereas no difference in the clearance rate of either Ox- or AcLDL from the bloodstream was found between the KO and wild-type mice (Ling et al. 1997). In ApoE or LDL receptor null mouse models of atherosclerosis, deletion of SR-A was found to significantly reduce the size of atherosclerotic lesions (Suzuki et al. 1997; Sakaguchi et al. 1998; Babaev et al. 2000). However, Moore et al reported recently that ApoE-KO mice deficient in SR-A or CD36 did not show a decrease in atherosclerosis (Moore et al. 2005). They found, in contrast, that male ApoE/SR-A-double KO mice showed an increase of atherosclerotic lesion area. The reasons for these different results are not known, but the different outcomes might be influenced by differences in the genetic background of the mice, differences in examination time-points after atherogenic feeding, and so on (Witztum 2005). In any case, these contradictory data reflect the complexity of the role of SR-A in atherogenesis, and indicate that additional studies are needed to clarify its role. SR-A has also been implicated to be involved in several other pathophysiologic processes, such as Alzheimer’s disease and diabetics, where SR-A binds to β-amyloid protein and AGE-modified proteins, respectively (El Khoury et al. 1996; Horiuchi et al. 2005). However, the role of SR-A in Alzheimer’s disease has been questioned since deletion of SR-A did not affect amyloid plaque formation or neurodegeneration in transgenic mice expressing human amyloid protein precursors (Huang et al. 1999). Based on the
finding that macrophages from SR-A-KO mice showed 50% reduction in the phagocytosis of apoptotic thymocytes, it was proposed that SR-A may play a role in the removal of apoptotic cells (Platt et al. 1996). However, no significant defects in thymocyte clearance were seen in the KO mice in vivo (Platt et al. 2000). Further, macrophages in the interdigit region of 13.5-embryonic mice express SR-A at high levels, suggesting that SR-A is actively involved in apoptotic cell clearance during embryonic morphogenesis (Komohara et al. 2005). However, SR-A is not essential for the embryonic clearance of apoptotic cells, because SR-A-deficient embryos develop normally without any retardation in footplate remodeling. Interestingly, CD36 was found to be upregulated in the SR-A-deficient fetal macrophages, suggesting that it substitutes for the SR-A function (Komohara et al. 2005). To conclude, SR-A might be involved in several physiological or pathophysiological processes through binding and phagocytosis of modified self-antigens, but, possibly due to functional redundancy between SR-A and other scavenger receptors or other type of receptors, it per se may not be crucial for tissue homeostasis or disease development.

Several studies indicate that SR-A and MARCO recognize also unmodified endogenous molecules. The first finding in this regard was by Gordon and coworkers who isolated the now well-known rat antibody 2F8, which was found to block cation-independent adhesion of macrophages to tissue culture plastic in the presence of serum (Fraser et al. 1993). This antibody was found to recognize an epitope in SR-A. Similarly, in a macrophage adhesion assay to frozen tissue sections, the antibody blocked the EDTA-resistant adhesion, indicating the presence of tissue ligands (Hughes et al. 1995). Further, SR-A-KO macrophages display impaired spreading ability in culture (Suzuki et al. 1997), and transfection of SR-A can enhance the adhesion property of the weakly adhering HEK 293 cells (Robbins & Horlick 1998). SR-A can interact with several proteoglycans, and these interactions may contribute to the adhesion of macrophages to the extracellular matrix (Santiago-Garcia et al. 2003). In addition, SR-A has been shown to mediate binding and uptake of gp96/GRP94 and calreticulin by APCs, suggesting a role for SR-A in the regulation of cellular responses to heat shock proteins (Berwin et al. 2003).

MARCO, too, appears to play a role in adhesion and spreading processes. Indeed, ectopic MARCO expression in nonmyeloid cell lines was found to induce cell
morphological changes with the formation of large lamellipodia-like structures and dendritic cellular processes (Pikkarainen et al. 1999). Another study has shown that MARCO expression on the spleen MZ macrophages contributes to the retention of the MZ B cells. Evidence for this arose from the specific binding of soluble MARCO to these cells, and inhibition of the binding with an antibody against MARCO. The ligand of MARCO on the MZ B cells has not been identified (Karlsson et al. 2003).

7.2.2. Role of MARCO and SR-A in Host Defense

As already mentioned previously, both MARCO and SR-A bind Gram-negative and -positive bacteria. Both receptors have been shown to interact with LPS, the major surface component of Gram-negative bacteria (Hampton et al. 1991; Dunne et al. 1994; Sankala et al. 2002). SR-A has also been shown to bind LTA, the major surface component of Gram-positive bacteria (Dunne et al. 1994). Further, it has been shown to bind a mutant strain of Neisseria meningitides lacking LPS, indicating that LPS and LTA are not the only bacterial ligands of SR-A (Peiser et al. 2002). Along this line, SR-A was recently found to recognize cord factor, a glycolipid component in the cell wall of the intracellular pathogen Mycobacterium tuberculosis (Ozeki et al. 2006).

In addition to microbial components, MARCO and SR-A recognize unopsonized environmental particles, such as TiO$_2$, Fe$_2$O$_3$, SiO$_2$ and latex beads (Kobzik 1995; Palecanda et al. 1999). MARCO appears, in fact, to be the major receptor on lung alveolar macrophages (AM) for these particles. This notion is based on the work by Kobzik and coworkers, who showed in their initial studies about 10 years ago that particle binding is sensitive to general SR inhibitors, and also that the binding is largely retained in the SR-A-KO AMs. They generated an antibody that blocked binding of unopsonized particles to hamster AMs and this antibody was found to be directed against MARCO (Kobzik 1995; Palecanda et al. 1999). Recently, a similar approach was used to show the major role of MARCO for particle binding in human AMs (Arredouani et al. 2005).

Studies with the SR-A- and MARCO-KO mice have provided direct evidence that these SRs play a protecting role in host defense. SR-A-KO mice were more susceptible than their wild-type controls to infection with Gram-positive bacteria Listeria monocytogenes and Staphylococcus aureus (Suzuki et al. 1997; Thomas et al.
2000), as well as with the viral pathogen HSV-1 (Suzuki et al. 1997). The study with S. aureus indicated that opsonin-independent bacterial phagocytosis is significantly decreased in macrophages lacking SR-A, and this might be the reason for the susceptibility of the SR-A-KO mice to infection with this bacterial strain (Thomas et al. 2000). BCG (Bacille Calmette-Guerin)-primed SR-A-KO mice were highly susceptible to a subsequent LPS challenge with increased production of TNF-α and IL-6 (Haworth et al. 1997). Another study showed that the SR-A-KO mice are more resistant than wild-type mice to LPS due to decreased secretion of IL-1β (Kobayashi et al. 2000). Yet, these two LPS-challenging studies are not comparable because of the differences in the challenging protocols. Arredouani et al challenged the MARCO- and SR-A-KO mice intranasally with Streptococcus pneumoniae or TiO₂ particles, and found diminished bacterial and particle clearance by the KO AMs, which resulted in increased inflammatory responses, and, in the case of pneumococcal infection, also to diminished survival (Arredouani et al. 2004; Arredouani et al. 2006). In conclusion, these studies demonstrate the importance of class A SR expression on AMs in responses against air-borne pathogens and inhaled particles.

Regarding bacterial clearance in the lung, MARCO appears to exhibit not only direct but also opsonin-mediated binding of bacteria. This notion is based on the finding that MARCO was identified as a receptor for UGRP1 (Uteroglobin-related protein 1), a small secreted protein expressed by epithelial cells of the bronchioles that is capable for bacterial and yeast binding (Bin et al. 2003).

As the studies cited above indicate, MARCO and SR-A clearly play a role in bacterial or particle clearance, and their absence can affect local inflammatory responses. Several studies have utilized the SR-A- and MARCO-KO mice to address the question whether deletion of these receptors affects cell signaling in the receptor-expressing macrophages themselves. Peiser et al showed that although SR-A is an almost exclusive phagocytic receptor for unopsonised Neisseria meningitides on bone marrow-derived macrophages, induction of proinflammatory cytokines depends on TLR4 but not on SR-A (Peiser et al. 2002). Zhu et al found that although SR-A binds bacterial DNA and CpG oligonucleotides, it is not essential for the uptake of DNA into cells or for cytokine induction (Zhu et al. 2001). Freeman and colleagues demonstrated that the signaling pathways activated by fucoidan and LTA, which were in previous studies claimed to be dependent on SR-A (Hsu et al. 1998; Pollaud-
Cherion et al. 1998; Whitman et al. 2000; Coller & Paulnock 2001; Hsu et al. 2001), were actually retained in the SR-A-KO macrophages, but were largely abrogated in the mice lacking CD14 (Kim et al. 2003). In contrast, when mice were challenged with an intraperitoneal injection of thioglycollate (TG), lack of SR-A was found to lead to increased secretion of the proinflammatory chemokines MIP-2 and KC (keratinocyte-derived chemokine), as well as to increased recruitment of neutrophils to the peritoneal cavity. Increased chemokine production was also seen in the peritoneal macrophages of the KO mice in vitro following exposure to TG. It was found to depend on particle internalization, and was also detected when the cells were exposed to latex beads coated with a SR-A ligand AGE-BSA, but not when exposed to soluble AGE-BSA (Cotena et al. 2004). Ozeki et al treated cultured AMs and Kupffer cells from the SR-A-KO and wild-type mice with the M. tuberculosis cord factor, and found, with the exception of MIP-1α production in Kupffer cells, increased production of TNF-α and MIP-1α in the SR-A-KO cells (Ozeki et al. 2006). Instead, when challenged with LPS, the KO Kupffer cells, but not the AMs, showed enhanced production of these factors. Finally, Jozefowski et al stimulated thioglycollate-elicited peritoneal macrophages from wild-type, SR-A- and MARCO-KO mice with LPS, or with LPS plus IFN-γ, and found that the SR-A-KO cells produced more and the MARCO-KO cells less IL-12 than their wild-type counterparts (Jozefowski & Kobzik 2004; Jozefowski et al. 2005). Further, ligation of MARCO with a specific antibody was found to cause an increase in IL-12 production in wild-type cells, demonstrating that ligand engagement directly induces intracellular signaling. In another study, addition of the SR-A-specific antibody 2F8 inhibited LPS/IFN-γ-stimulated IL-12 production in both alveolar and peritoneal macrophages (Jozefowski & Kobzik 2004; Jozefowski et al. 2005). Moreover, immobilized 2F8 was found to stimulate $\text{H}_2\text{O}_2$ production in both cell populations without the presence of LPS/IFN-γ (Jozefowski & Kobzik 2004). Thus, it appears that the functions of MARCO and SR-A may extent beyond the ligand clearance function.
AIMS OF THE PRESENT STUDY

At the time this study started, MARCO-KO mice had already been generated in our group. These mice appeared normal in a pathogen-free environment. However, *in vitro* studies had indicated that MARCO might be involved in host defense by recognizing pathogenic antigens (Elomaa *et al.* 1995; Palecanda *et al.* 1999), as well as in tissue homeostasis by promoting cell adhesion and changes in cell morphology (Pikkarainen *et al.* 1999). The primary goal was to study whether these proposed functions are real functions of MARCO using MARCO-deficient mice. Secondly, we wanted to generate MARCO/SR-A-double-KO mice to study combined effects of the MARCO and SR-A gene ablations. We were particularly interested in studying the effects of the gene ablations on the structure and function of the spleen MZ, because this is one of few locations where MARCO is normally expressed, and SR-A is also expressed at this location. Furthermore, it is known that this spleen compartment has an important role in host defense functions.

Being a PRR, MARCO is able to recognize conserved molecular patterns. Characterization of its ligand-binding properties and identification of new ligands are important research topics, which will contribute to the understanding of its biological roles. Motivated from this point of view, a search for novel ligands of MARCO and characterization of its binding properties became another important aim of this study.
MATERIALS AND METHODS

1. Animals and animal experiments (paper I, III)
Mice deficient for MARCO (MARCO-KO) or SR-A (SR-A-KO) were backcrossed to the C57BL/6 (B6) strain for more than 10 generations. The MARCO-KO mice were mated with the SR-A-KO mice to produce MARCO/SR-A double-KO mice. Control wild-type mice were from the breedings of the MARCO heterozygous mice. All mice appeared normal and fertile in a pathogen-free environment. All mouse studies were approved by the regional ethical committee for experimental animals.

1.1. Macrophage Isolation and culture (paper I, III)
Bio-gel-elicited peritoneal macrophages were prepared by an intraperitoneal injection of 1 ml of polyacrylamide gel P-100 (Bio-Rad) beads in endotoxin-free water (2% bead solution). After 4 days, peritoneal cells were isolated by lavage with PBS. Thioglycollate-elicited macrophages were isolated 4 days after an intraperitoneal injection of 1 ml of 3% Brewer’s thioglycollate (Sigma). Resident peritoneal cells were isolated from untreated mice by rinsing the cavity with DMEM containing 10% FCS or with PBS. Macrophages were isolated from other cell types by exploiting their ability to strongly adhere to glass or plastic: cells were plated on tissue-culture dishes for at least 2 h, after which the dishes were washed three times with PBS to remove non-adhered cells.

When studying the effects of the gene deletions on cell spreading, resident peritoneal cells were cultured in DMEM/FCS on glass coverslips for 2 h or longer. For cell counting, freshly isolated peritoneal cells were cytopspun onto microscope slides, dried, fixed, and visualized by DAPI nucleic acid dye staining. Macrophages were visualized by the F4/80 mAb staining. The number of F4/80-positive cells divided by the number of DAPI-positive cells represented the proportion of macrophages in the total cell population. For measuring TNF-α and NO responses to Neisseria meningitides (NM) stimulation, macrophages were cultured in serum-free OPTIMEM (Invitrogen) containing different doses of NM, in the presence or absence
of 20 ng/ml of IFN-γ for 24 h. The culture supernatants were harvested, TNF-α and NO release was measured by ELISA (Pharmingen) and the Greiss reaction (Sigma), respectively.

1. 2. Macrophage depletion in mice (paper I)
Liposome-entrapped dichloromethylene diphosphonate (clodronate) suspension was obtained from Clodronateliposomes (Free University, Amsterdam, The Netherlands). To deplete cells from the spleen, 0.2-ml aliquots of the suspension were injected intravenously into each mouse. Clodronate is taken up by phagocytic cells, such as macrophages, where it causes rapid apoptosis. Reappearance of macrophages and the other MZ cell populations was monitored 4, 8, 11, 16, 21, 35, and 67 days after the treatment, by staining frozen sections with the various antibodies described below. At least two mice per genotype were examined at each time point. This experiment was repeated twice.

1. 3. Antibody responses to pneumococcal polysaccharides (paper I)
Pneumo23, a 23-valent pneumococcal vaccine containing 25 µg of the capsular polysaccharides from Streptococcus pneumoniae serotypes 1–5, 6B, 7F, 8, 9N, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F in 0.5 ml of NaCl with 0.25% phenol, was obtained from Aventis Pasteur. A 500 µl sample of the vaccine diluted 1/25 in 0.9% NaCl was injected intraperitoneally to 5–9 mice per genotype in each experiment. The experiment was performed twice. Blood samples were taken by puncturing the tail artery at days 0, 7, 14, and 63 after the immunization from the same mice over time. Sera were stored at -20°C until analyzed. Serum anti-Pneumo23 IgM and IgG3 Igs were measured by ELISA.

ELISA plates were coated with 100 µl of the vaccine at the concentration of 2.3 µg/ml PBS for 2 h at 37°C. After washing four times with PBS/0.05% Tween 20 (PBST), the wells were incubated in 1% BSA to block the remaining binding sites. Then, 100 µl of the serum dilution (diluted in PBST containing 0.5% BSA) was applied, and the anti-vaccine antibodies were allowed to bind for 2 h at 37°C or overnight at 4°C. When testing anti-vaccine IgM, we used a serum dilution 1/1000. In case of anti-vaccine IgG3, a serum dilution of 1/10 was used. These were found to be the proper dilutions in pilot experiments. Each serum sample was tested in duplicate.
All bleedings were tested simultaneously. After the incubations, the wells were washed several times with PBST and incubated with biotinylated rabbit anti-mouse IgM (Zymed Laboratories) or biotinylated monoclonal rat anti-mouse IgG3 (BD Pharmingen) for 90 min at 37°C. After washings with PBST, the wells were incubated in HRP-conjugated streptavidin (Pierce; diluted 1/10 000 in PBST) at room temperature (RT) for 20 min, and then washed again. A substrate solution sample of 100 µl, a 1:1 mixture of reagent A (H₂O₂) and reagent B (tetramethylbenzidine) (R&D Systems), was added into each well, and the color was allowed to develop for 10–20 min. The reaction was terminated by adding 50 µl of 2 N H₂SO₄. Absorbance values were read in a microplate reader at 450 nm and corrected by values obtained at 570 nm.

2. Immunofluorescent staining (paper I, II, III)
Fresh OCT-embedded tissues were frozen in liquid nitrogen and stored at -70°C. Cryosections of 8 µm were fixed in acetone for 10 min. Cells cultured on glass coverslips were washed with PBS, and fixed with 4% paraformaldehyde.

After incubation in 10% normal serum from the species in which the secondary Ab was generated, the tissue sections were incubated with the primary Ab, followed by several washes in PBS and incubation with a fluorescently-labeled secondary Ab. Cells cultured on coverslips were permeabilized in 0.1% Triton X-100/PBS for 5 min, and incubated in 2% BSA before staining. For double staining, tissue sections were first stained for one of the Ags, then incubated in 20% normal rat serum again, and subsequently stained for the other Ag by first incubating with a biotinylated mAb and then with fluorescently-labeled streptavidin. The following rat anti-mouse mAbs were used: ED31, an anti-MARCO mAb; ERTR9, a mAb against SIGNR1 expressed on the MZMs; MOMA-1, recognizing Siglec-1 expressed on the MMMs; MECA367, an anti-MAdCAM-1 mAb staining the endothelial cells lining the MZ sinus (all these antibodies were kindly provided by Georg Kraal, Free University, Amsterdam); F4/80 (clone CI:A3-1; Serotec), a pan-macrophage marker; anti-IgD mAb (clone 11-26; Southern Biotechnology Associates). Additionally, biotinylated rabbit anti-mouse IgM (µ-chain specific; Zymed Laboratories) was used. The anti-IgD and -IgM Abs were used to identify the MZ B cells (IgM<sup>high</sup>/IgD<sup>low</sup>). Binding of the unconjugated primary Abs was detected with Alexa Fluor 488- or 546-
conjugated goat anti-rat Abs (Molecular Probes). Biotinylated primary Abs were detected with Alexa Fluor 594-conjugated streptavidin (Molecular Probes) or with FITC-conjugated streptavidin (DakoCytomation). The cell nucleus and the actin cytoskeleton were visualized, respectively, by DAPI staining (Molecular Probes), and staining with rhodamine-conjugated phalloidin (Molecular Probes).

3. Migration assay (paper I)
Migration activity of resident peritoneal macrophages was assayed using the Transwell two-chamber system (Costar, 8-µm pore size, 6.5-mm insert diameter). Resident peritoneal cells were harvested with PBS, washed once with DMEM containing 0.2% BSA and 15 mM Hepes (pH 7.4), and resuspended at 0.5 x 10⁶ cells/ml in the same medium. 100 µl of a cell suspension was applied into the upper chamber. The lower chamber contained 10% FCS in DMEM (600 µl). After incubation for 5 h at 37°C, cells on the upper side of the membrane were removed with a cotton tip and three rinses with PBS. Cells on the underside of the membrane were fixed with methanol overnight at 4°C, and stained with F4/80 and DAPI. The membranes were mounted on glass slides, and cells were counted under a fluorescence microscope with an x20 objective. To evaluate the number of peritoneal macrophages in the cell suspension applied into the upper chamber, an aliquot of the cell suspension was plated for 5 h on a glass coverslip, after which the coverslip was rinsed twice with PBS, and the attached cells were fixed in methanol and stained with F4/80 and DAPI.

4. Surface Plasmon Resonance (SPR) experiments (paper II)
Surface plasmon resonance (SPR), a highly specialized optical technique offering the unique opportunity to observe molecular interactions in real-time, was applied to study those of the recombinant soluble MARCO protein, sMARCO. All SPR experiments were run at 25 °C at a flow rate of 5 µl/min in PBS or 10 mM Hepes, pH 7.4, 150 mM NaCl, using a BIAcore 3000 instrument and NTA sensor chips (BIAcore AB). Buffers were degassed and filtered through 0.2-µm cutoff filters. Prior to an experiment, purified sMARCO (ligand) in the eluent buffer (PBS) was coupled to the flow cells at densities ranging from 1000 to 3000 RU. Flow cells without immobilized sMARCO were used as reference cells. Analytes were injected over the flow cell.
surfaces at the following concentrations: LPS and LTA, 25–100 µg/ml; poly(I) and heparin, 5–15 µg/ml. The LPS and LTA solutions were sonicated 3 times for 15 s before the use. The injection time was 7 min. A fresh ligand was applied to the flow cells before each run. Thus, after a run, the chip was washed with 250 mM EDTA, followed with 100 mM NaOH. Thereafter, the NTA surface was first recharged with nickel ions before applying sMARCO. The control flow cell was not loaded with sMARCO. This reloading procedure was chosen, because we observed that a fraction of sMARCO was stripped away from the NTA acid surface when LPS and LTA were passed over the chip.

5. Phage display screen (paper II)

A complex phage display library was screened to identify short MARCO-binding peptides. As a first step, we coated a high concentration of sMARCO onto a well of a Nunc Maxisorp plate (coating with 100 µg/ml sMARCO in PBS overnight at 4 °C). After blocking 1–2 h in 2% BSA/PBS at RT, the phage library solution (1 x 10^9 transducing units (TU) in 2% BSA/PBS) was added, and the plate was incubated for 2 h at RT. The well was washed with PBST to remove unbound phages. Bound phages were eluted with a low pH buffer, neutralized, and used to infect competent K91kan E. coli. Three more rounds of panning were carried out in the same manner, except that less sMARCO was coated onto the microtiter plates for rounds three and four (50 and 500 ng/well), and the phage solution was incubated with the immobilized sMARCO for 1 h only. During these rounds, enrichment was verified by comparing the phage binding onto sMARCO- and BSA-coated surfaces. Randomly selected clones were sequenced.

Binding of individual phages to surfaces coated with sMARCO, recombinant SRCR domain of MARCO, recV, or recombinant nephrin encompassing the first two IgG domains (rNephrin) was tested in the same manner (1 x 10^8 TU of phages added per well). Proteins were coated at the concentration of 10 µg/ml overnight at 4 °C (100 µl/well). Two wells were coated with each protein. Control wells were coated with a similar concentration of BSA. The assay was repeated three times. Similar assays were also performed in the presence of GST, GST-VRWGSFAAWL (the most enriched phage peptide fused to GST), or a synthetic VRWGSFAAWL peptide (Anaspec).
6. Production of the GST-VRWGSFAAWL peptide fusion protein (paper II)

The construct encoding the GST-VRWGSFAAWL peptide fusion protein was generated as follows. A fragment encoding the phage insert was first produced by PCR with the forward and reverse primers containing, respectively, BamHI and EcoRI recognition sites (the forward primer: 5'-GGCTCGAGGATCTCGGCCGACGGGGCT-3', the reverse primer: 5'-AGGTCTAGAATTCGCCCCAGCGGCCCC-3) using phage DNA as a template. The fragment was gel-purified, digested with BamHI and EcoRI, and cloned into the BamHI-EcoRI-digested pGEX-2TK. The correctness of the construct was verified by DNA sequencing. The GST fusion protein and GST alone were expressed in E. coli BL21 strain. The proteins were produced and purified according to the manufacturer’s instructions (Amersham Biosciences).

7. Various MARCO-expression constructs and transient transfections (paper II, III)

All MARCO expression constructs used in transient transfections were cloned into the mammalian expression vector pcDNA3 (Invitrogen). DNA manipulations were carried out using established molecular biological methods. A construct encoding truncated mouse MARCO lacking the SRCR domain was created by replacing the region encoding the C-terminal part of MARCO by a fragment encoding a stop codon after the codon for serine 419, the last residue of the collagenous domain. The form “Minicollagen” has, as the name indicates, a very short collagenous domain, containing only the first 8 Gly-X-Y repeats of the 89-repeat-long domain. Constructs encoding chimeric SCARA5 proteins were generated by replacing the entire SRCR domain of SCARA5, or portions of the domain, with corresponding segments of MARCO. The form IW contains the MARCO segment 423-481 (from the beginning of the MARCO SRCR domain to tryptophan 481). The form NC contains MARCO residues 423-507 (from the beginning of the MARCO SRCR domain to cysteine 507).

CHO cells were transfected using the calcium-phosphate method. Precipitates containing calcium phosphate and DNA were first formed in a Hepes-buffered saline solution, and then applied on cells. 20 µg of DNA was used per a 100-mm dish. After overnight incubation, the cells were washed with PBS, and fed with fresh medium.
They were seeded on glass coverslips 24 h after the transfection. The binding assays were performed next day.

8. Binding assays (paper II, III)
For the binding of DiI-labeled AcLDL and FITC-labeled bacteria, transfected cells were first washed once with DMEM/10-20 mM Hepes, pH 7.5 (DMEM/Hepes), and then incubated in DMEM/Hepes containing either 2.5 µg/ml of AcLDL or different concentrations of bacteria for 60 min in a humidified atmosphere with 5% CO₂ at 37°C, in the presence or absence of poly (I) or poly (C). Thereafter, the cells were washed two times with DMEM/Hepes, and two times with PBS before fixation with 4% paraformaldehyde.

For the binding of phage clones, the GST proteins and human complement proteins C4 and C4b (Advanced Research Technologies), the cells were first incubated for 10 min in ice-cold DMEM/Hepes containing 2% BSA (when testing the binding of the purified proteins, BSA was not always included). After removal of this solution, a similar solution containing a test component (phage, 1 x 10⁹ TU; a GST protein, 100 µg/ml; C4 and C4b, 50 µg/ml) was added, and the incubation on ice was continued for 45–60 min. Cells were washed five times with PBS, and fixed as above. After fixation, the binding was visualized by immunofluorescent staining and evaluated under fluorescent microscope. Phage binding was detected by an anti-M13 mAb (Amersham Biosciences) (10 µg/ml). To detect the binding of the GST proteins, both commercial (Amersham) and homemade anti-GST antibodies were used. The C4- or C4b-binding was visualized by an anti-human complement protein C4 antibody (Sigma). In some assays, we tested the binding of recombinant human C4d that represents a physiological cleavage fragment of C4b. The fragment was biotinylated, and the binding was detected with FITC-labeled streptavidin. Many of the binding assays with C4, C4b, and C4d were performed in the presence of GST or GST-VRWGSFAAWL.

Cell-free bacteria-binding assays were carried out on glass coverslips coated with 5 µg of sMARCO or a control protein, recombinant nephrin. The glass coverslips were coated with the proteins for 1 h at RT, or overnight at 4°C. The coverslips were then incubated with 1 mg/ml BSA in PBS for 30 min at RT and washed three times with 20 mM Tris-HCl, pH 7.5, 0.1 M NaCl. Fluorescent-labeled
bacteria were added in the absence of serum and incubated for 1 h at 37°C. In some
assays, coverslips were preincubated with 50 µg/ml of poly (I) or poly (C) for 30 min.
The bindings were evaluated under fluorescent microscope.
RESULTS

Analysis of MARCO- and MARCO/SR-A-Double-KO mice indicates a role for MARCO in tissue homeostasis and immune response (paper I)

Several previous observations have pointed to the possibility that lack of MARCO, SR-A, or both of these proteins may affect the structure of the spleen MZ. First, the macrophages in the spleen MZ are one of few MARCO-expressing cell populations in a pathogen-free mouse (Elomaa et al. 1995). Second, ectopic expression of MARCO has been found to induce cell spreading and formation of dendritic cellular processes in nonmyeloid cell lines (Pikkarainen et al. 1999). Third, SR-A is also expressed at high levels by the MZ macrophages. Fourth, there are indications that SR-A mediates cell adhesion in vivo through binding of endogenous ligands (Hughes et al. 1995; Santiago-Garcia et al. 2003). We therefore investigated whether genetic ablation of these two class A SRs affects macrophage morphology and/or their positioning in vivo. By using antibodies recognizing the different MZ cell populations, we studied the impact of the gene deletions on the MZ structure in adult mice in steady-state, as well as during the ontogenic development of the spleen, and the recovery process after depletion of the spleen MZ cell populations in adult mice. In sum, we found that the development of the spleen MZ was delayed during ontogeny and after cell depletion in mice lacking MARCO and both MARCO and SR-A, and that its microarchitecture was still immature at adult age.

The MZ develops after birth, and staining of spleen sections from wild-type mice at the day of birth showed the presence of large numbers of MARCO-positive cells dispersed throughout the whole spleen. The MARCO-positive MZ pattern started to appear at day 3 after birth, having a clear pattern at day 7. The expression of another MZM marker, SIGNR1, which largely colocalizes with MARCO in adult mice, was not detected in wild-type mice before day 7. Similarly, the expression of the MMM marker Siglec-1 was not detected in wild-type mice before day 7 after birth. A more typical ring-like MZ staining with these two markers was only seen at about day 12 in wild-type mice. Notably, expression of these two markers was never
detected outside the MZ. Analysis of the KO mice indicated that their expression was markedly delayed in the absence of MARCO or both MARCO and SR-A.

The development of the MAdCAM-1-positive MZ sinus was found to coincide with that of the MARCO-positive MZ in wild-type mice. Similarly to the MARCO-expressing cells, MAdCAM-1-positive cells were found dispersed throughout the spleen at the day of birth. In wild-type mice, the MAdCAM-1-positive MZ pattern started to appear at day 3, but this was delayed until day 7 in the MARCO-KO and day 9 in the double-KO mice.

The F4/80 mAb is often used as a pan-macrophage marker, but it does not stain the MARCO-positive MZMs in adult mice. Notably, the expression of MARCO and F4/80 are not overlapping at the day of birth either, indicating that the MZ and the red pulp macrophages are different macrophage populations already during the ontogenic development of the spleen. When staining spleen sections from adult mice for SIGNR1, the MZ structure was, as mentioned already above, still found to be immature in the KO mice. There were fewer numbers of the MZMs in the KO mice, and these were more sparsely distributed than those in wild-type mice. These findings were verified by acid phosphatase staining, a method identifying macrophages based on their enzyme activity, irrespective of receptor expression (Kraal 1992). To explore whether the rehabilitation of splenic macrophages from bone marrow precursors is also defective in the KO mice, we used the clodronate-liposome treatment model. An intravenous injection of clodronate-liposomes leads to a rapid depletion (within 24 hours) of the spleen red pulp and MZ macrophages, while the white pulp macrophages stay intact. The red pulp macrophages normally reappear from bone marrow precursors within 1 week and the MZ cells later (MZ B cells and MAdCAM-1 expression also transiently disappear) (van Rooijen et al. 1989). Altogether, the reappearance process was very similar to that seen during the ontogenic development of the MZ. Thus, MARCO-positive cells reappeared first in the red pulp and migrated to the MZ within the next week. Expression of SIGNR1 and Siglec-1 was detected only after the formation of the MARCO-positive MZ, and only in the MZ. As was their appearance delayed in the KO mice during ontogeny, it was delayed during this recovery process too. For example, while the staining for SIGNR1 and Siglec-1 showed a clear MZ pattern at day 35 after treatment in wild-type mice, these two markers only started to appear in the KO mice at this time-point. A similar delay was
also seen in the reappearance of MAdCAM-1 expression and the MZ B cells. However, the development of MZ B cells was not affected in the KO mice during ontogeny and at adult age.

The spleen plays a major role in the protection against infections of encapsulated bacteria, such as *S. pneumoniae* (Gopal & Bisno 1977; Amlot & Hayes 1985). The capsular polysaccharides (PSs) are TI-2 antigens against which the MZ B cells produce antibodies to provide protection (Kraal 1992; Guinamard *et al.* 2000). We observed an impaired response in the KO mice when injected with a pneumococcal polysaccharide vaccine Pneumo23, a TI-2 antigen. We measured anti-Pneumo23 IgM and IgG3 production in mouse serum at 1, 2 and 9 weeks after injection of a single dose of vaccine (Perlmutter *et al.* 1978; Sarvas *et al.* 1983; Shapiro *et al.* 1998). The KO mice showed lower IgM and IgG3 responses compared to wild-type mice. The double-KO mice showed the lowest response.

In pathogen-free mice, the lymph node medullary macrophages and the resident peritoneal macrophages are besides the MZMs the other MARCO-expressing macrophage populations (Elomaa *et al.* 1995). As was the case with the MZMs, the KO mice had significantly reduced numbers of resident peritoneal macrophages. However, MARCO does not seem to play a role in the recruitment (migration) of new macrophages to the peritoneum, since in the thioglycollate-induced peritonitis model, the newcomers were not found to express MARCO. Furthermore, in a two-chamber migration assay, resident peritoneal macrophages from wild-type mice did not show an increased migratory ability compared with the corresponding KO cells. On the contrary, we found 2-3-fold or more of the double-KO cells migrated into the lower chamber in this assay. This result supports the observation that lack of MARCO affects the retention of macrophages in the peritoneal cavity as well as in the spleen MZ, which causes a reduction in the size of these two macrophage populations in the KO mice. We also examined the spreading capability of these cells. Indeed, the resident peritoneal macrophages from the KO mice exhibited an impaired spreading property. *Figure 3* shows scanning electron microscopic pictures of cells cultured overnight on tissue-culture plastic. Wild-type macrophages spread well with numerous dendritic processes, which are very few in the KO cells.
Generally, all the above-described phenotypes were more striking in the double-KO mice than in mice lacking only MARCO or SR-A, which indicates a role for both of these class A SRs in tissue homeostasis and responses against TI-2 antigens.

**Phage display screen and binding assays with AcLDL confirm the crucial role of the SRCR domain in the ligand binding function of MARCO (paper II)**

Previous work indicated that the SRCR domain is of major importance for the bacteria-binding capability of MARCO, as well as for its capability to induce formation of dendritic cellular processes (Brannstrom et al. 2002; Pikkarainen et al. 1999). Here, further evidence was provided for the notion that the SRCR domain is the major ligand-binding domain in MARCO.

This work is largely based on sMARCO, a recombinant protein produced in the mammalian 293-cell expression system (Sankala et al. 2002). When immobilized on a glass coverslip, the protein was able to bind *E. coli*. In another assay, LPS was found to interact with beads conjugated with sMARCO, but not with control beads (Sankala et al. 2002). Here, we studied the binding characteristics of sMARCO further, and examined its interaction with LPS, LTA and poly (I) in real time using the BIACore system. We found that all these polyanionic compounds interact with sMARCO, but poly (I), a macromolecule blocking bacterial binding to MARCO-expressing cells, has clearly the highest affinity. An indication of selectivity was that the fourth polyanionic compound tested, heparin, which does not affect bacterial binding to cells expressing MARCO, did not interact with sMARCO in this system.
With the primary aim of identifying novel physiological ligands of MARCO, we then immobilized a high concentration of sMARCO to a plastic surface, and used this surface to capture phage clones from a random, linear decapeptide M13-phage library. Altogether, four rounds of selection were performed. From round four, 5 different sequences were recovered out of 31 clones sequenced. Contrary to our expectations, all these sequences had a hydrophobic character instead of a polyanionic one. The most enriched peptides, VRWGSFAAWL and RLNWAWWLSY, were displayed on 20 and 5 clones, respectively. Only these two clones were studied further. First, we confirmed the interaction of the VRWGSFAAWL phage with sMARCO in the BIAcore system, where it was found to bind with a higher affinity than LPS and LTA.

Database searches with the VRWGSFAAWL peptide sequence suggested an intriguing possibility that complement component C4 may be a ligand of MARCO. For example, when the peptide was analyzed against human proteins in the NCBI databank, this complement component gave the second highest hit (a 7-residue continuous match GSFAAWL). However, we could not convincingly demonstrate that C4 is a ligand of MARCO. Although we could detect binding of C4b (the activated form of C4) and C4d (a physiological degradation fragment of C4b) to full-length MARCO transfectants, the binding could not be inhibited by a high molar amount of GST-VRWGSFAAWL. An antibody against the phage peptide also failed to recognize C4b and C4d in ELISA and in Western blots.

Several results indicated that the VRWGSFAAWL and the RLNWAWWLSY phage bind to the SRCR domain of MARCO. Thus, cells expressing full-length MARCO, but not those expressing truncated MARCO lacking the SRCR domain, bound the phage clones. In a cell-free system, we assayed phage binding to microtiter wells coated with BSA, sMARCO, recV (recombinant SRCR domain of MARCO) or rNephrin (recombinant nephrin). When comparing plating dilutions giving only 1-2 colonies on the rNephrin-plate, there were about 200 colonies on the recV-plate, and more than 1000 colonies on the sMARCO-plate. These results not only indicate that it is the SRCR domain that contains the phage binding site(s), but also that there might be a difference in the strength of interaction between phage and the trimeric sMARCO or the monomeric SRCR domain. However, we cannot exclude the possibility that there are less phage-binding sites accessible on the surface coated with
the small recV molecule than on that coated with sMARCO. GST-VRWGSFAAWL, but not GST alone, was also found to bind transfectants expressing full-length MARCO. As expected, GST-VRWGSFAAWL did not bind to cells expressing the truncation without the SRCR domain. Further, since all above-described assays were performed with mouse MARCO and its derivates, we wanted to test whether GST-VRWGSFAAWL is recognized by MARCO from another species. Indeed, it was found to bind equally well to cells expressing full-length human MARCO, whose SRCR domain has 74 % sequence identity with that of mouse MARCO. This finding demonstrated that the interaction is not species-specific. On the other hand, cells expressing the mouse MARCO truncation extending 17 residues to the SRCR domain did not bind the fusion protein, although they exhibited avid bacterial binding. In competition studies, GST-VRWGSFAAWL, as well as a synthetic VRWGSFAAWL peptide, were able to block the binding of both phage clones to immobilized sMARCO and recV. This suggests that the phage clones may bind to the same site on MARCO.

With the aim of mapping the peptide-binding site in the SRCR domain, we generated constructs encoding chimeric SRs. The ‘backbone’ in these constructs was the newly discovered member of the class A SR subfamily, SCARA5, whose sequence has been published by two groups (Sarraj et al. 2005; Jiang et al. 2006), but the receptor has also been cloned in our group (J. Ojala and K. Tryggvason, unpublished data). We first showed that cells expressing this novel SR did not bind GST-VRWGSFAAWL. We then replaced the entire SRCR domain of SCARA5, or portions of the domain, with the corresponding segments of mouse MARCO, and tested the capability of these chimeric proteins for GST-VRWGSFAAWL binding. The chimera “IW” contains the MARCO SRCR sequence from valine 423 to tryphophan 481 (in mouse MARCO, residues 420-518 encompass the SRCR domain), whereas the form “NC” contains the MARCO segment 423-507 (cyesteine). We did not expect that the chimeras with an ‘incomplete’ MARCO SRCR domain bind GST-VRWGSFAAWL better than the one with the complete MARCO SRCR domain, but this was the case. These results may indicate that the SRCR domains of the receptors have conformational differences. For AcLDL binding, the receptors showed very different activities. Cells expressing the chimera with the complete MARCO SRCR domain bound AcLDL avidly, whereas those expressing the forms IW and NC bound
this prototypic SR ligand very weakly. In case of bacterial binding, no significant differences were found between these forms. First of all, these findings strongly indicate AcLDL-binding activity for the SRCR domain of MARCO. Second, it seems that this activity, but not the bacteria-binding activity, is sensitive to minor structural alterations.

To confirm the role of the SRCR domain for AcLDL binding in MARCO, we assayed AcLDL binding to different mouse MARCO transfectants. The transfectants expressing full-length MARCO, or the form “Minicollagen”, whose collagenous domain consists only of the first 8 Gly-X-Y repeats of the 89-repeat-long domain (but with an intact SRCR domain), bound AcLDL very well, while the form lacking the SRCR domain did not exhibit AcLDL binding. Thus, the SRCR domain appears to be crucial for AcLDL in MARCO.

**MARCO, an innate activation marker of macrophages, recognizes Neisseria meningitides independently of LPS (paper III)**

It has been shown that SR-A is a major PRR for phagocytosis of Neisseria meningitidis (NM) in bone-marrow derived macrophages. On the other hand, it is only partially involved in the uptake of other bacteria, such as E. coli. In Bio-gel-elicited peritoneal macrophages, the contribution of SR-A for NM binding is much less (Peiser et al. 2000; Peiser et al. 2002). It has also been shown that SR-A can recognize NM independently of LPS. This study was primarily set out to examine the contribution of MARCO for NM recognition in two macrophage populations, Bio-gel-elicited and resident peritoneal macrophages.

First, we utilized both sMARCO and transfectants expressing either full-length mouse or human MARCO to show that MARCO recognizes wild-type NM. Next, we tested the interaction of a NM mutant strain that completely lacks LPS (strain lpxA). Interestingly, this strain was well recognized by MARCO. The binding of both wild-type and the mutant NM was inhibited by poly (I), a high-affinity ligand of MARCO (paper II). In contrast, the binding was unaffected by poly (C), a polyanion that is not a SR ligand. In sum, these studies show that although MARCO clearly recognizes LPS, or at least its soluble form (paper II), additional ligand(s) are displayed on the surface of NM.
In further studies, the binding of NM, \textit{E. coli} and AcLDL to the Bio-gel-elicited and resident peritoneal macrophages from wild-type, the MARCO-, the SR-A-, and the double-KO mice was quantitated by flow cytometry. The results indicated that SR-A contributes more than MARCO to the binding of these 'ligands' in these two cell populations. In case of each ligand, the double-KO cells exhibited the lowest binding. The contribution of MARCO for the ligand binding was higher in the resident peritoneal macrophages than in the Bio-gel-elicited cells, which is well in line with the fact that the former population expresses higher levels of MARCO than the latter one (paper I). Finally, the cells from wild-type and the KO mice were incubated with different concentrations of NM in the presence or absence of IFN-\(\lambda\), and the production of TNF-\(\alpha\) and NO in culture supernatants was measured after 24 h of incubation. IFN-\(\lambda\) significantly increased the production of both TNF-\(\alpha\) and NO in all cell populations tested, but lack of neither SR-A nor MARCO affected their production.
DISCUSSION

The major finding of this thesis work is that scavenger receptor MARCO, as well as its relative SR-A, are important for the development of the spleen MZ. These studies have also shed light on the ligand-binding characteristics of MARCO, providing further evidence for the notion that the SRCR domain has a major role in MARCO’s ligand-binding function. Moreover, the role of MARCO and SR-A in host defense against the Gram-negative bacterium *Neisseria meningitides* was studied. Both receptors contribute to its recognition, possibly through both LPS-dependent and -independent recognition mechanisms. However, deletion of MARCO and/or SR-A was not found to affect the production of TNF-α or nitric oxide upon bacterial stimulation.

**Developmental Role of MARCO and SR-A**

A developmental role of MARCO and SR-A was revealed in the studies of the MARCO- and MARCO/SR-A-double-KO mice. Using markers for the various MZ cell populations, a significant delay was found in the development of the spleen MZ during ontogeny and after MZ cell depletion in adult mice in the absence of MARCO and both MARCO and SR-A. Moreover, the MZ microarchitecture was still immature at adult age.

The MZMs were traced by staining for SIGNR1, a transmembrane lectin expressed on the surface of these cells. It is notable that SIGNR1 expression was not detected outside the MZ, but it was induced only after the characteristic ring-like MZ pattern of MARCO was very obvious. It thus appears that the localization of the MARCO-positive cells to the MZ precedes their further differentiation. Thereafter, other factors are required. Indeed, in mice defective for M-CSF expression (op/op mice), SIGNR1 and the MMM marker Siglec-1 are not expressed, whereas the MARCO-positive MZMs are present (Ito et al. 1999).

Analysis of adult mice in steady-state indicated more scattered and reduced numbers of SIGNR1-positive cells in the KO mice. These findings point to the
possibility that MARCO and SR-A are needed not only for the development but also for the maintenance of the proper MZ microstructure. Similarly, we observed reduced numbers of resident peritoneal macrophages in the KO mice. Analysis of resident peritoneal macrophages in vitro indicated defects in cell spreading and cell retention, providing a possible explanation for the observed phenomena.

Our findings add to the growing evidence that there exists a complex interplay between the various MZ cell types, including the MZMs, the MMMs, the MZ B cells and the sinus-lining cells. Ablation of genes such as those genes encoding TNF, p55TNF-R, RelB, and Bcl-3 have been found to result in phenotypes where the numbers of the SIGNR1-positive MZMs are reduced drastically, and MAdCAM-1 is either completely missing or expressed at a very low level (Franzoso et al. 1997; Poljak et al. 1999; Pasparakis et al. 2000; Weih et al. 2001). Similarly, mice lacking the transcription factor NKX2.3, which is required for MAdCAM-1 expression, were found to have reduced numbers of the Siglec-1-positive MMMs and SIGNR1-positive MZMs (Pabst et al. 2000). In addition, there clearly is interplay between B cells and macrophages. The absence of signaling through lymphotoxin on B cells led not only to a reduction in the size of the MZ B cell-population, but also to that of the SIGNR1-, Siglec-1-, and MAdCAM-1-positive cell populations (Tumanov et al. 2002). Related to this finding, a recent study with several KO models revealed that B cells are important both for the development and the maintenance of the MZ macrophage subpopulations (Nolte et al. 2004). Another recent study has shown that the interaction between MARCO and a cell surface determinant on the MZ B cells contributes to the retention of the MZ B cells within the MZ (Karlsson et al. 2003). This is in contrast to our ontogeny study where the lack of MARCO and SR-A was not found to affect the appearance of the MZ B cells, but it is in line with our liposome depletion study, which argues for a role of MARCO in the retention of the MZ B cells. It may be the case that the importance of this interaction for the MZ B cell retention is not uncovered in the chronic loss-of-gene function situation, but only in an acute situation, such as the recovery process after the liposome treatment and anti-MARCO antibody treatment (Karlsson et al. 2003).

The KO mice exhibited a clearly impaired response against a pneumococcal polysaccharide (PS) vaccine, a TI-2 antigen. After an intravenous injection, TI-2 antigens are rapidly captured in the spleen by the MZMs and MZ B cells via,
respectively, SIGNR1- and complement-mediated processes (Kraal et al. 1989; van den Eertwegh et al. 1992; Guinamard et al. 2000). The MZ B cells are responsible for the rapid production of protective antibodies (Kraal 1992; Guinamard et al. 2000). The role of the MZMs in this regard is not certain, but there are indications that the MZ B cell responses to TI-2 antigens depend on the proper microenvironment generated by spleen macrophage-like cells (Garg et al. 1996; Balazs et al. 2002). This view is supported by the finding that the impaired responses of spleen cells from aged mice could be restored by supplementation with various cytokines (Garg et al. 1996). It is also notable that there is an age-associated decline in antibody responses to the pneumococcal PS in human as well as in mice. It is of interest in this regard that we have observed reduced numbers of the MZMs in aged mice by staining for MARCO and SIGNR1 (data not shown). Increased susceptibility to infections with encapsulated bacteria is also seen in human young infants under 2 years of age and rodent pups under 3-4 weeks old, whose MZ has not yet fully matured (Timens et al. 1989; Takeya & Takahashi 1992; Kruschinski et al. 2004). Furthermore, splenectomized patients are at risk for developing severe infection, especially with these encapsulated bacteria (Gopal & Bisno 1977; Amlot & Hayes 1985).

Besides the MZ B cells, peritoneal B1 B cells participate in the early immune responses against TI antigens (Martin et al. 2001). The size of this cell population was very similar in wild-type and the KO mice (data not shown). However, peritoneal macrophages have been shown to effectively support B1 B-cell differentiation in TI responses (Balazs et al. 2002), and therefore it is possible that the reduction in the size of the peritoneal macrophage population contributes to the impaired TI-2 response in the KO mice.

**Novel Insights into the Ligand-binding Function of MARCO**

The phenotypic defects seen in the spleen and peritoneal cavity of the KO mice suggest that MARCO and SR-A affect cell positioning through interaction with local structural components. The MARCO-MZ B cell interaction is another indication that UGRP1 (Bin et al. 2003) is not the only unmodified endogenous ligand of MARCO. Moreover, as we show in this thesis work, MARCO avidly binds a *Neisseria* strain completely lacking LPS, which suggests it can recognize microbial surface protein(s).
With these indications in mind, we searched for novel ligands of MARCO by employing the unbiased phage display method. Although this study did not lead to the identification of novel protein ligands of MARCO, it nevertheless provided new significant insights into its ligand-binding characteristics. Database searches suggested that the most enriched peptide, VRWGSFAAWL, represents complement component C4, but we could not convincingly confirm this suggestion experimentally. Moreover, the crystal structure of the C4d fragment indicates that the side chains of many of the residues in the proposed binding site are not exposed, which argues against the possibility that C4 is a ligand of MARCO. Consequently, it is not clear at the moment which molecule the phage peptide(s) represent(s). In this regard, one has to keep in mind that it is not uncommon that an isolated peptide is only a structural mimic of the ligand and does not bear high enough sequence similarity to what by the BLAST search. This problem might possibly be overcome with the help of anti-peptide antibodies (Cardo-Vila et al. 2003). We generated antibodies against the VRWGSFAAWL peptide, but they have not so far been useful tools for the ligand identification.

The results of the phage display screen and the experiment that followed the screen can be summarized as follows. (a) They revealed the capability of MARCO to recognize peptides with a predominantly hydrophobic character. (b) The SRCR domain was found to be responsible both for the phage binding and AcLDL binding. These results thus strengthen the importance of this domain as a ligand-binding domain of MARCO. (c) The results also provide evidence suggesting that even minor structural alterations in the SRCR domain can have profound effects on AcLDL binding. Concerning the data on AcLDL, it is worth reminding that for SR-A, a cluster of basic residues at the C-terminal end of the collagenous domain has been indicated as the AcLDL-binding site (Acton et al. 1993; Doi et al. 1993). Similar cluster is also present in MARCO. No function has been assigned for the SRCR domain of SR-A.

Based on the present knowledge of the scavenger receptor ligands, we expected that, if obtaining any enrichment in the phage display screen, polyanionic peptides might be enriched. Surprisingly, no polyanionic peptides were enriched. The isolated peptides were not found to have extensive sequence identity, but they all have a basic residue near or at the N-terminal followed by a stretch of mostly hydrophobic
residues. The BIAcore analysis showed that the VRWGSFAAWL phage interacted with sMARCO with slower dissociation kinetics than LPS or LTA.

The localization of phage and AcLDL binding activities to the SRCR domain adds to the increasing evidence that the SRCR domain is the major functional domain in MARCO (Elomaa et al. 1998; Brannstrom et al. 2002). The AcLDL-binding activity, as well as the activities mapped to this domain in previous studies, are inhibitable by poly (I) but not by GST-VRWGSFAAWL, indicating a polyanionic nature for the interacting ligands. However, it is worth mentioning that although GST-VRWGSFAAWL did not inhibit AcLDL binding to MARCO, a synthetic VRWGSFAAWL peptide had a marked inhibitory effect. If the blockage is not due to the binding of soluble peptide aggregates, this finding may indicate that the peptide and AcLDL bind to the same or overlapping sites on the SRCR domain. The crystal structure of the recombinant SRCR domain, recV, has been solved in our group (J. Ojala, manuscript in preparation), and the structure indicates a cluster of arginine residues, which may be important for the binding of AcLDL. We have also tried to crystallize the VRWGSFAAWL peptide/recV complex, but the complex has a low peptide occupancy, possibly due to the low solubility of the peptide in aqueous solutions. In any case, we can observe extra electron density between two of the arginines. This supports the view that the binding sites of the peptide and AcLDL are at least partially overlapping. Whatever is the case, this domain appears to have multiple binding interfaces, because the mode of the interaction of these two ligands with MARCO has to be quite different. Related to the presence of the arginine cluster, it is of interest to note that the long side chain of arginine can be involved both in polar and nonpolar interactions. Thus, while the guanido group is involved in ionic interactions, the rest of the side chain may be involved in hydrophobic interactions (Andrew et al. 2001).

The binding experiments with the chimeric scavenger receptors demonstrated that AcLDL binding is surprisingly sensitive to even very minor changes in the primary structure of SRCR domain. Thus, the replacement of the last 11 residues of the MARCO SRCR domain with the corresponding sequences from SCARA5 had a dramatic effect on AcLDL binding. Neither the C-terminal 11-residue segment of MARCO, VHNEDAGVECS, nor that of SCARA5, GHAEDAGVTCTVP, contains any positively charged residues, which are considered to be crucial for the interaction
with AcLDL. This suggests that the sequence replacement affects AcLDL binding indirectly by causing a structural change. This view is supported by the results from the binding studies with GST-VRWGSFAAWL. However, differences in AcLDL binding cannot be due to any major structural alterations, because the C-terminal segment of both MARCO and SCARA5 contains a cysteine residue, which stabilizes the position of the very C-terminal end by an intradomain disulfide bond. Instead, examination of the crystal structure of the MARCO SRCR domain suggests that the single amino acid changes may alter the conformation of arginine residues in a six-stranded β-sheet in which the C-terminal segment AGVECS participates (J. Ojala, unpublished information), thereby affecting AcLDL binding, and that of the GST fusion protein, too.

**Role of MARCO and SR-A in Anti-microbial Host Defense**

The results from the bacteria-binding assays with Bio-gel-elicited and resident peritoneal macrophages indicated that both MARCO and SR-A contribute to the recognition of *N. meningitides* and *E. coli* in these cell populations. Both receptors are able of binding soluble LPS, but they can recognize *N. meningitides* independently of this major outer membrane component. It remains to be seen whether the receptors recognize different ligands, or whether the affinities differ for a same ligand. Notably, although lack of MARCO and SR-A markedly affected bacterial recognition, no differences in the production of TNF-α and nitric oxide were seen when the peritoneal macrophages from the different genotypes were stimulated with wild-type *N. meningitides*. In this regard, it is worth pointing out that there is some evidence suggesting that lack of SR-A promotes TNF-α production. Absence of SR-A was found to lead to increased production of TNF-α and IL-6 in BCG-primed mice stimulated with LPS (Haworth *et al.* 1997). In another study, stimulation of alveolar macrophages and Kupffer cells with the SR-A ligand *M. tuberculosis* cord factor lead to enhanced production of TNF-α in the absence of SR-A expression (Ozeki *et al.* 2006). Similarly, Kupffer cells from the SR-A-KO mice produced more TNF-α in response to LPS stimulation than the corresponding wild-type cells (Ozeki *et al.* 2006). Taken together, it appears that the concept proposing that removal of ‘nonsignaling’ scavenger receptors promotes TNF-α production by providing more
ligand for the signaling PRRs does not hold true for \textit{N. meningitides}, a complex ‘ligand’, and peritoneal macrophages.
REFERENCES


